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Partition of proteins in an aqueous phase system of dextran and polyethylene glycol

Influence of the electrolyte content

By P. Å. Albertsson and Ed. J. Nyns

With 6 figures in the text

The distribution of proteins, viruses, and cell particles in aqueous polymer two-phase systems has recently been described [1–7]. These phase systems are obtained by dissolving two different polymers in water. Studies on the behaviour of proteins [1, 2] and virus particles [3, 4, 5, 6] have shown that these distribute in a reproducible manner, giving rise to a finite partition coefficient.

The partition coefficient depends on the nature of the phase system and the size and properties of the particle surface of the partitioned substance. It has been found that in a dextran-methylcellulose system the partition coefficient depends mainly on the particle size and the distribution is only slightly affected by the nature of the electrolytes present. In contrast, a dextran-polyethylene glycol system shows very striking salt effects. Thus, for example, a protein may be transferred almost entirely from one phase to the other by changing the electrolyte composition.

This salt effect is of great theoretical interest and has also been used for practical purposes such as fractionation of proteins by counter-current distribution [2] and the concentration of viruses [5]. In the study of immunological reactions in two-phase systems a proper choice of the electrolyte composition is also necessary to obtain the desired property of the phase system [8].

It was therefore considered worth while to study this salt effect in more detail and this paper presents a study of the distribution of some selected proteins in a dextran-polyethylene glycol system as a function of the electrolyte content.

Materials and methods

Dextran was obtained from Pharmacia Ltd. (Uppsala, Sweden). A fraction, "Dextran 500", with a limiting viscosity number, 48 ml/g; weight average molecular weight, 460,000; and number average molecular weight, 180,000 (according to the manufacturer), was used throughout the experiments. The dextran is first wetted and allowed to swell in water overnight and then dissolved by gentle boiling. If the solution is boiled too long or too hard, a slightly brown colour appears, giving an absorption maximum at about 260 mμ. For some experiments, the dextran solution was

reduced by $NaBH_4$ [9] and the dextran precipitated with alcohol. The precipitate was washed with 95 % alcohol and dried in air. This purified dextran is soluble in cold water and has a lower UV absorption (particularly around 260 mμ) than the original product. The concentrations of the dextran solutions were determined by polarimeter, assuming $[d]_D^{20} = +199°$.

Polyethylene glycol was obtained from Carbon and Carbide Chemicals Co., U.S.A., as Carbowax polyethyleneglycol 6000; it will be referred to as PEG 6000. It has, according to the manufacturer, a mean molecular weight of 6000–7500. It contains an impurity that absorbs in the UV at 290 mμ [10] and which may be removed by precipitating the PEG from an acetone solution with ether. The concentrations of PEG solutions were determined by weighing, after having dried the solution first by freeze-drying and then over P_2O_5 in a desiccator for one day.

Phycoerythrin and phycocyanin were obtained from *Ceramium rubrum* according to Kylin [11] and purified by twice-repeated chromatography on calcium phosphate according to Tiselius *et al.* [12]. The phycoerythrin eluted with 0.01 molar phosphate buffer pH 6.8 was used. Although this component was already freed from coloured impurities by calcium phosphate chromatography, two peaks appeared on ultracentrifugation, a minor one moving slowly and not coloured and a major one moving quickly and containing all the colour. By electrophoresis two components again appeared, one not moving and the other moving with the colour. This last component was slightly heterogenous, though the heterogeneity was due to minor components present at concentrations just detectable by the method. The phycoerythrin was thus not pure but could be considered so practically from the colour point of view. Its concentration was determined by extinction measurements at 540 mμ, assuming the extinction of a 1 per cent w/v solution to be 62.2, or at 495 mμ, assuming the extinction of a 1 per cent w/v solution to be 60.4. The phycocyanin used was that eluted from a calcium phosphate column with 0.06 *M* phosphate buffer of pH 7.0. It was found to be homogeneous by free electrophoresis and ultracentrifugation. Its concentration was determined by absorption at 615 mμ assuming the extinction of a 1 per cent w/v solution to be 44.

Ceruloplasmin was obtained from Kabi Ltd., Stockholm, Sweden. Its concentration was determined by its oxidase activity [13].

Serum albumin (human) was obtained from Kabi Ltd., Stockholm. Electrophoretically, the product contained 98 % albumin and in the ultracentrifuge all the material sedimented as one peak. The concentration of serum albumin was determined by the Kjeldahl micro-method.

Barley albumin was obtained by water extraction of a Kenya 1955 barley flour according to Rondelet & Lontie [14]. Barley albumin was determined in the top phase by the biuret test according to Weichselbaum [15].

All salts used were of analytical grade.

A partition experiment was carried out as follows. A stock mixture was prepared by mixing the polymer solutions and water in such proportions that, when the mixture is diluted with the protein and salt solutions, the desired phase system is obtained. For example, a stock mixture may contain 14 per cent (w/w) dextran, 8.8 % (w/w) PEG and 77.2 % (w/w) water. This mixture was shaken and a sample immediately withdrawn using a pipette with a wide orifice. 5 g of this mixture were then quickly weighed into a separating funnel together with 5 ml of a solution of protein and salt. The tube was inverted about 50 times and thermostated at 20.0°C. After 1 hour for phase separation, samples were taken for protein determination.

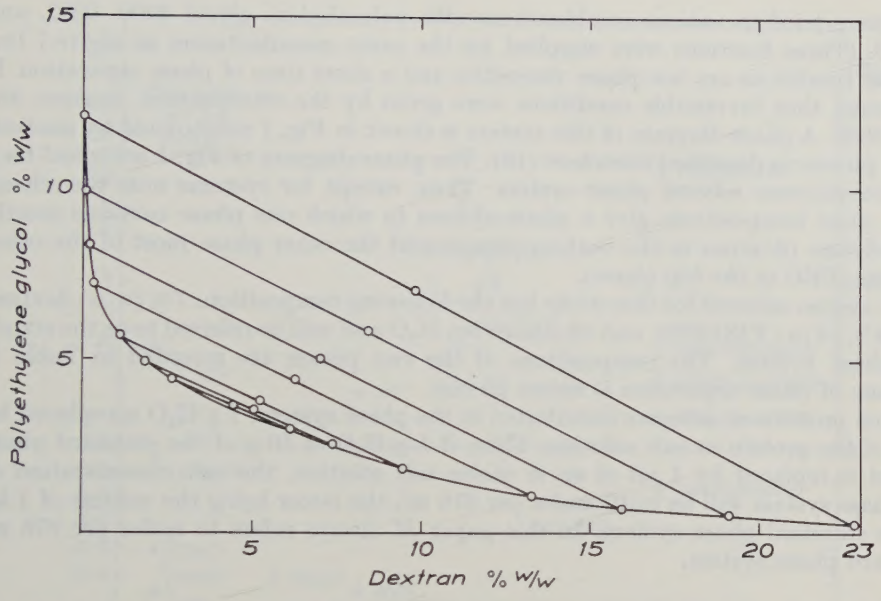

Fig. 1. Phase diagram of a two-phase system of dextran-polyethylene glycol-water at 20°C.

For extinction measurements 1 g of a phase was diluted with at least 1 ml H_2O. Because of the high viscosity of the polymer solutions and phases, these are measured by weighing in order to get accurate results. The top phase of the phase system has, however, such a low viscosity that samples of this phase may be taken with a pipette.

The phase system

It has been shown elsewhere [1, 2] that the partition coefficient depends on the molecular weight of the phase polymers in such way that the proteins have more affinity for fractions of lower molecular weight. To have phase systems with a suitable range of partition coefficients ($0.1 < K < 10$) for this investigation, preliminary experiments were made with different fractions of dextran and polyethylene glycol. Thus dextran fractions with limiting viscosity numbers of 17, 24, 37, 48, and 68

Table 1. The composition of the phases in a dextran-polyethylene glycol-water system at 20°C.

Total composition: 7% (w/w) dextran: 4.4 % polyethylene glycol: 86.6 % (w/w) H_2O. Volume ratio top phase/bottom phase = 1.08.

	Dextran, % w/w	Polyethylene glycol, % w/w	H_2O, % w/w
Bottom phase	14.2	0.9	84.9
Top phase	0.20	7.65	92.15

ml/g were tried in various combinations with polyethylene glycol 4000, 6000, and 20,000. (These fractions were supplied by the same manufacturers as above.) Important conditions are low-phase viscosities and a short time of phase separation. It was found that favourable conditions were given by the combination, dextran 48–PEG 6000. A phase diagram of this system is shown in Fig. 1 constructed by analysis of the phases as described elsewhere (16). The phase diagram of Fig. 1 is typical for a polymer-polymer solvent phase system. Thus, except for systems near the critical point, most compositions give a phase system in which one phase contains mostly one polymer (dextran in the bottom phase), and the other phase most of the other polymer (PEG in the top phase).

The system selected for this study has the following composition: 7 % (w/w) dextran 48, 4.4 % (w/w) PEG 6000, and 88.6 % (w/w) H_2O and will be referred to as the standard phase system. The compositions of the two phases are recorded in Table 1; the time of phase separation is about 20 min.

When proteins or salts are distributed in the phase system, 1 g H_2O is replaced by 1 ml of the protein or salt solution. Thus, if 1 g H_2O of 10 g of the standard phase system is replaced by 1 ml of an m molar salt solution, the salt concentration of the phase system will be m/10 moles per 976 ml, the latter being the volume of 1 kg of the standard phase system. In this paper M always refers to moles per 976 ml standard phase system.

Results

A. *The influence of different salts on the distribution of phycoerythrin*

The distribution of phycoerythrin in the presence of 0.01 M potassium phosphate buffer pH = 6.8 and different salts is shown in Fig. 2. In the presence of the buffer alone, the protein is mainly in the top phase with a K value of about 7. Addition of other salts correspondingly gave definite K values. All K values thus obtained were lower than that obtained in the buffer alone. This tendency of salts to lower the K value is very different for the various salts tested. Thus ammonium sulfate and citrate hardly affect the K value while $NaNO_3$ reduces it from 7 to about 0.3 so that the major part of the protein is transferred to the bottom phase.

There seems to be a specific mechanism regarding the ability of the different salts to lower the K values. As may be seen in Fig. 2 and Table 2, the potassium halides lower the K value in the following order $F^- < Cl^- < Br^- < I^-$, and the alkaline chlorides in the order $Li^+ < NH_4^+ < Na^+ = Cs^+ < K^+$.

As Table 3 shows, the difference in effect of the ammonium and sodium ions remains the same whether the anion is chloride, nitrate or sulphate. The difference in the effect of the two ions does not therefore seem to depend on the other ions present.

B. *Influence of the concentration of ions on the distribution of phycoerythrin*

In a first series of experiments potassium phosphate buffer was used alone. Three experiments are recorded in Fig. 3.

In the first experiment (Fig. 3*a*) the concentration of K_2HPO_4 was kept constant at 10^{-2} M, and the concentration of KH_2PO_4 increased from 10^{-4} to 10^{-2} M. As may be seen in Fig. 3*a*, the K value remains constant up to 10^{-3} M KH_2PO_4 and then decreases.

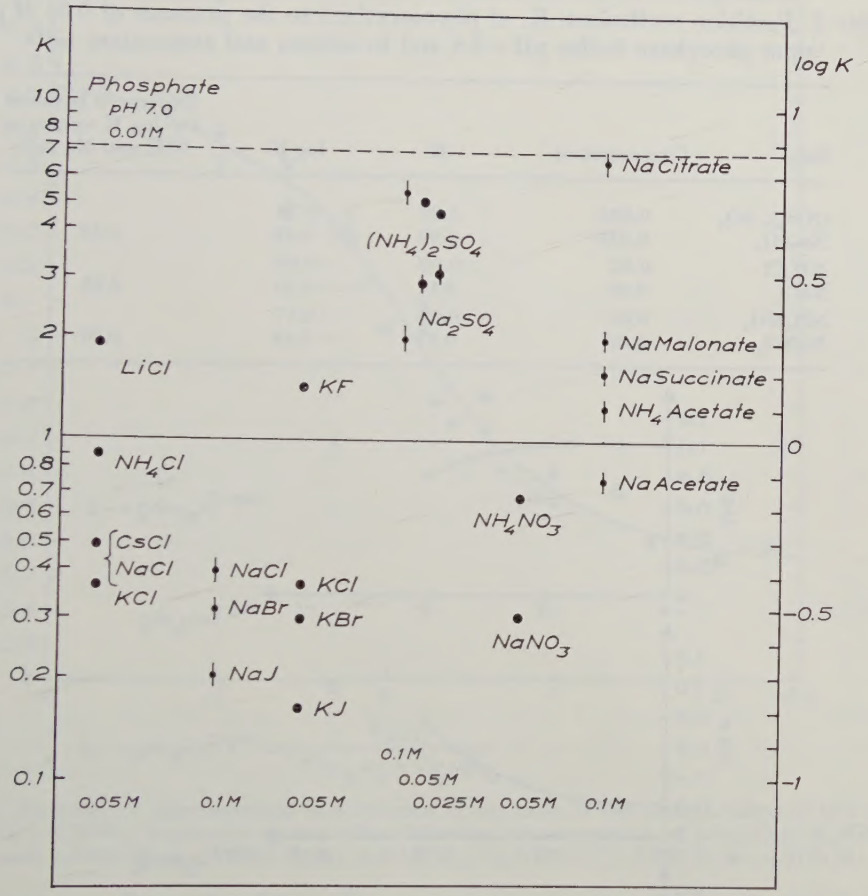

Fig. 2. Partition coefficient, K, of phycoerythrin in a dextran-polyethylene glycol phase system with 0.01 *M* potassium phosphate buffer, pH = 6.8, and with different salts; the concentrations of the latter are given in the lower part of the figure.

Table 2. Partition coefficient (K) of phycoerythrin in a dextran-polyethylene glycol system in the presence of 0.01 *M* potassium phosphate buffer pH = 7.0 and 0.05 *M* of the indicated salt.

Alkali chlorides			Potassium halogenides		
Salt	K	log K	K	K	log K
—	7	0.85	—	7	0.85
LiCl	1.91	0.28	KF	1.4	0.16
NH_4Cl	0.90	−0.048	KCl	0.37	−0.44
NaCl	0.49	−0.31	KBr	0.28	−0.55
KCl	0.37	−0.44	KI	0.16	−0.80
CsCl	0.49	−0.31			

Table 3. Partition coefficient, K, of phycoerythrin in the precence of 0.01 *M* potassium phosphate buffer pH = 6.8 and in sodium and ammonium salts.

Salt	Concentration	K	log K	Difference between the log K values in NH_4 and Na salts
$(NH_4)_2\,SO_4$	0.025	5.09	0.71	
Na_2SO_4	0.025	2.99	0.48	0.23
NH_4Cl	0.05	0.90	−0.05	
NaCl	0.05	0.49	−0.31	0.26
NH_4NO_3	0.05	0.68	−0.17	
$NaNO_3$	0.05	0.37	−0.43	0.26

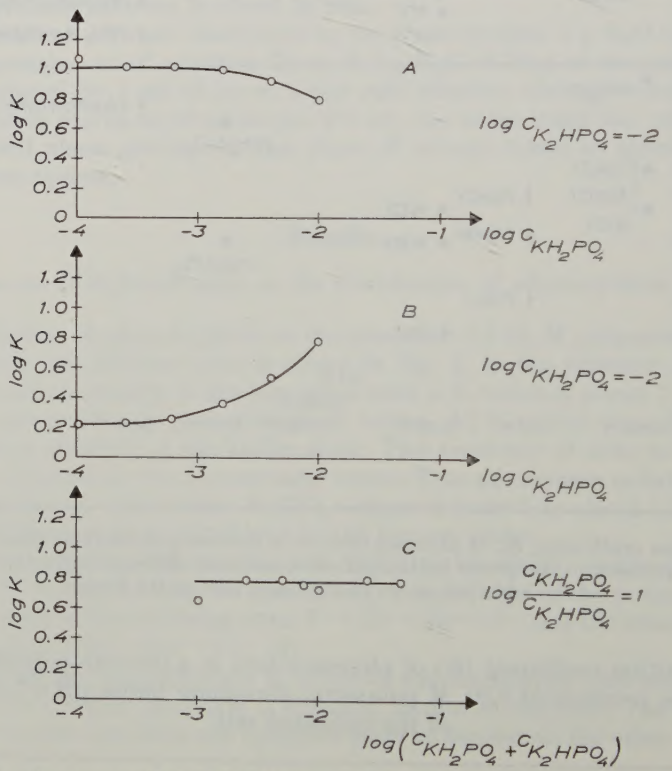

Fig. 3. Partition of phycoerythrin in a dextran-polyethylene glycol system with different concentrations of KH_2PO_4 and K_2HPO_4.

In the second experiment (Fig. 3*b*) the concentration of KH_2PO_4 was kept constant at 10^{-2} *M*, and the concentration of K_2HPO_4 increased from 10^{-4} to 10^{-2} *M*. As seen in Fig. 3*b*, the K value remains constant up to 10^{-3} *M* KH_2PO_4 and then increases.

In the third experiment (Fig. 3*c*) the ratio of the concentrations of the two phosphates was kept constant and equal to 1, while their concentrations were increased. In this case the K value remains almost constant.

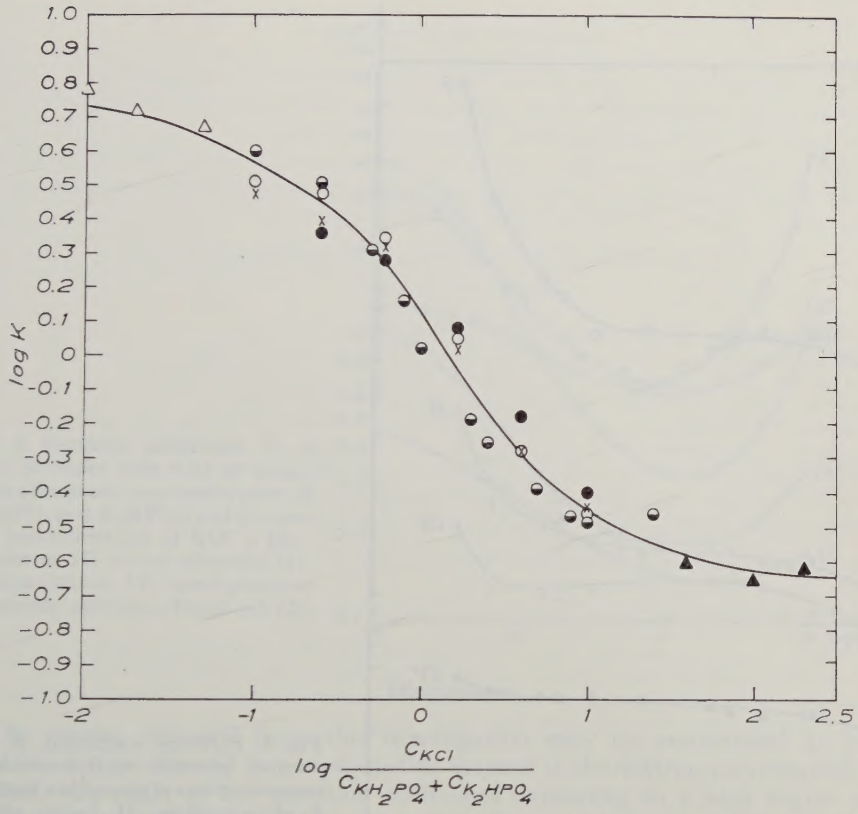

Fig. 4. Partition of phycoerythrin as a function of the ratio of the concentrations of KCl and $KH_2PO_4 + K_2HPO_4$ (equimolar parts). The following concentrations of $KH_2PO_4 (= K_2HPO_4)$ were used: 0.00025 M (▲), 0.0005 M (●), 0.00125 M (×), 0.0025 (○), 0.005 M (◒), 0.0125 M (△).

In summary, the results of these three experiments indicate that the K value is not affected by the addition of one phosphate up to less than 1/10 of the concentration of the other phosphate and that the K value remains constant at different salt concentrations below 0.1 *M* provided the ratio between the two phosphates remains the same.

In the second series of experiments the effect of added KCl on the K value of phycoerythrin in phosphate buffer was studied. In each experiment the concentration of the phosphate buffer was kept constant and the KCl concentration varied from 1/100 to 100 times that of the phosphate. The results are shown in Fig. 4 where the log K values have been plotted against the ratio of the concentrations of KCl and phosphate. As may be seen in Fig. 4, the different values gather around a sigmoid curve indicating that the K value is determined almost only by the ratio of the KCl and phosphate buffer concentrations. For a given ratio the K value is independent of the ionic strength up to 0.2 *M*. Fig. 4 also shows that if the one salt has a concentration of 10 times or more that of the other, then the K value is hardly affected by changes in the ratio of the two salts.

Thus, in both the cases, K_2HPO_4–KH_2PO_4 and KCl–K_2HPO_4, KH_2PO_4 at ionic strengths below 0.1 *M*, it is mainly the ratio of the salts which determines the partition coefficient.

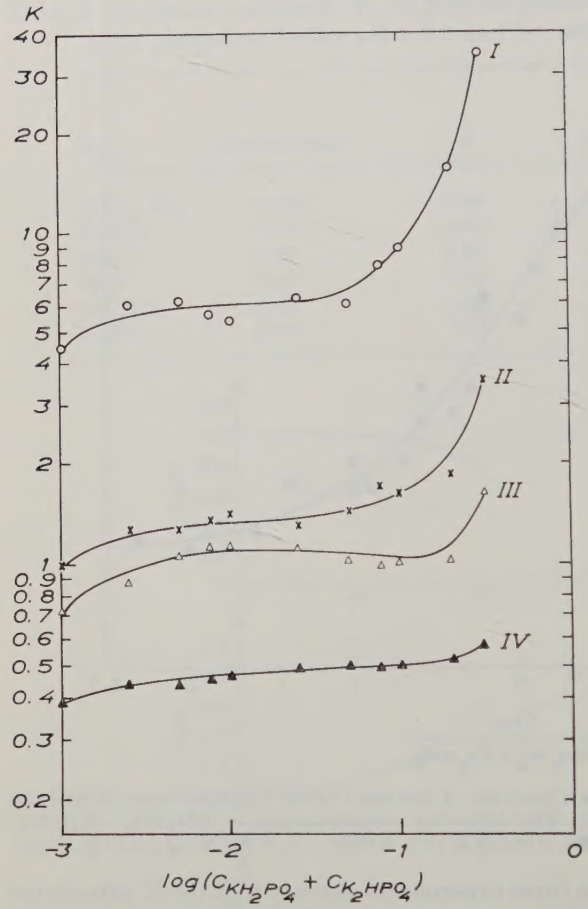

Fig. 5. Partition coefficient, K, of various proteins with increasing concentrations of phosphate buffer. I, phycoerythrin; II, barley albumin; III, ceruloplasmin; IV, serum albumin.

C. *Influence of the salt content on the distribution of different proteins*

So far we have described the influence of salts on the distribution of only one protein, phycoerythrin. For comparison, a number of different proteins have been studied. The results are shown in Figs. 5 and 6. The influence of increasing the concentration of phosphate buffer is shown in Fig. 5. The K value first increases somewhat and then it becomes almost constant between 0.004–0.1 *M* phosphate buffer. At higher concentrations the K values increase considerably. The influence of increasing the concentration of KCl at constant buffer concentration, is shown by Fig. 6. This experiment corresponds to that described in Fig. 4. The curves are of the same shape for the different proteins, and in all cases, the addition of KCl lowers the K value up to a KCl concentration of 0.1–0.2 *M*. At higher concentrations the K values increase. Quantitatively, the influence of the added KCl is different for the various proteins. Thus, the distribution of phycoerythrin is affected considerably, while for serum albumin and barley albumin it is affected to a smaller extent.

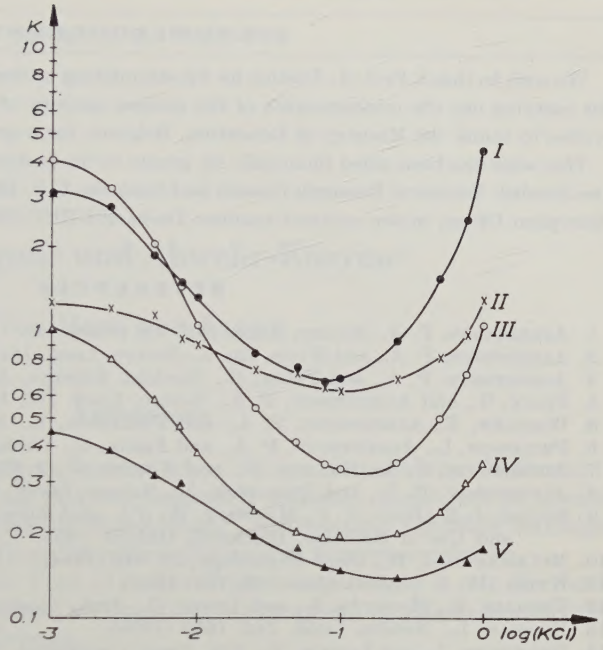

Fig. 6. Partition coefficient, K, of some proteins with 0.01 *M* potassium phosphate (equimolar parts of KH_2PO_4 and K_2HPO_4) and increasing concentrations of KCl. I, phycocyanin; II, barley albumin; III, phycoerythrin; IV, ceruloplasmin; V, serum albumin. From ref. [2].

Discussion

The results obtained from this investigation may be summarized as follows. Proteins are partitioned in a reproducible manner in the dextran-polyethylene glycol system, the value of the partition coefficient depending to a high degree on the electrolyte composition. In the concentration range between 0.001–0.1 *M*, it is chiefly the ratio of the ionic concentrations which determines the partition coefficient, while at a given ratio, the ionic strength has little influence. This is very convenient for practical purposes, since a suitable value of the partition coefficient may thus be obtained, over a wide range of ionic strengths, merely by varying the ratio of two ions. The fact that changes in the K value due to changes in the concentration of a salt are depressed when another salt is present in excess is also useful in practice. By carrying out a partition experiment with an excess of a neutral salt present, for example with 10 times higher NaCl concentrations than other salts, variations in certain minor electrolyte concentrations become negligible.

It has been shown elsewhere [16] that the partition of proteins in the phase system studied here is independent of the protein concentration up to fairly high concentrations and also, in all mixtures so far studied, independent of the presence of other proteins. In a defined salt medium, the partition coefficient of a protein in the dextran–polyethylene glycol system has therefore a fixed value which can be used for the characterization of a protein.

SUMMARY

The distribution of proteins in a liquid two-phase system composed of dextran, polyethylene glycol, water, and various electrolytes has been studied. With a defined salt medium reproducible partition coefficients can be measured. The experiments suggest that partition in the dextran polyethylene glycol system can be used for the characterization of proteins.

ACKNOWLEDGEMENTS

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Anti-Ergone und Abwehr-Enzyme

Von Hans von Euler

Antienzyme

Die Problematik der Antienzyme beginnt schon bei der Nomenklatur. Während in älteren Arbeiten Antienzyme als Enzyme behandelt wurden, macht sich in neuerer Zeit die Auffassung geltend, dass die Bezeichnung „Antienzyme"[1] Stoffen beliebiger chemischer Natur zukommt, die nicht notwendig zu den Enzymen gehören.

Eigentlich wären — wie der Verf. [1] schon früher betont hat — nur die durch parenterale Injektion von Enzymen im Organismus erzeugten, spezifisch hemmenden Stoffe als Antienzyme zu bezeichnen.

Zwischen Wirkstoffen und Anti-Wirkstoffen. Ergonen und Anti-Ergonen bestehen in chemischer wie auch in biologischer Hinsicht sehr mannigfache Beziehungen. So findet man unter den Anti-Ergonen, den Anti-Vitaminen, Anti-Hormonen sowie Anti-Wuchsstoffen fast alle wichtigeren Stoffklassen vertreten und ihre Gegen- und Hemmungs-Wirkungen beruhen auf ganz verschiedenen Reaktionsmechanismen. Bei den Anti-Vitaminen sind diese Mechanismen weitgehend aufgeklärt, während bezüglich der Antienzyme noch viele einander widersprechende Angaben vorliegen.

1. Antiurease

Die meisten und eingehendsten Arbeiten über Antienzyme beziehen sich auf die Antiurease, die nach Kirk und Sumner [2] beim Kaninchen und beim Huhn durch Injektion sehr kleiner Mengen krystallisierter Urease nach einer Latenzzeit von etwa 2 Monaten Immunität hervorruft. Die Ergebnisse von Kirk und Sumner sind von Pillemer u.a. [3] sowie von Howell [4] bestätigt worden.

Urease ist schon in Mengen von 0,3 mg/kg ein tötliches Gift (geprüft auch an Hund und Maus), und zwar durch die enzymatische Abspaltung von NH_3 aus dem Harnstoff des Blutes. In Sumners Arbeiten bildete sich nach wiederholter intraperitonealer Injektion sehr kleiner Mengen von Urease ein aus dem Blutserum isolierbarer Stoff, der die enzymatische Harnstoffspaltung hemmte und der ausserdem durch seine Fähigkeit, Urease zu fällen charakterisiert war.

Im Anschluss an Arbeiten über Tumor-Immunität haben wir frühere Untersuchungen des Institutes [5, 6, 7] über Antiurease und über andere Antienzyme wieder aufgenommen.

[1] Wir schreiben dementsprechend „Antienzyme" aber „Anti-Vitamine" und „Anti-Hormone", die nicht serologisch gekennzeichnet sind.

Material und Methodik zur Darstellung der Antiurease

Teils aus dem Pulver von Canavalia ensiformis (Schwertbohne; Jack bean), teils aus einem Präparat „Arlco"-Urease der Arlington Chem. Co., Yonkers, N.Y., wurden nach dem von Sumner angegebenen, von Dounce modifizierten Verfahren Urease-Präparate dargestellt.

Das verwendete „Arlco"-Urease-Präparat hatte 200 Urease-Einheiten/g. Urease-Einheit ist diejenige Urease-Menge, die bei 20°, pH 7 in 5 Min. 1 mg Ammoniak-Stickstoff freimacht. Die Aktivität der krystallisierten Urease wurde nach Euler und Brunius [8] gemessen durch die unter den gegebenen Versuchsbedingungen entwickelte Ammoniak-Menge.

Bezüglich der Darstellung, Reinigung und Aktivitätsbestimmung der Urease- und Antiurease-Präparate schliesst sich unsere Methodik an diejenige von Sumner an [9], die von diesem Forscher 1929 auch in unserem Stockholmer Institut verwendet worden war. Es kann also im Wesentlichen auf Sumners Angaben [2] verwiesen werden.

Von unserem „Arlco"-Pulver wurden zu jeder Versuchs-Serie 10 g in 250 ml wässriger Phosphat-Puffer-Lös. pH 7,4 gelöst und mit 120 ml Aceton unter Rühren versetzt. Die immer noch klare Lösung wurde mit Zitronensäure angesäuert bis Fällung eintrat, die nach 10 Min. abzentrifugiert und dann zur weiteren Verwendung in Wasser gelöst wurde; sie war als Urease hochaktiv.

Unsere an Ratten mit 11 Urease-Einheiten ausgeführten subkutanen und intraperitonealen Injektions-Versuche waren nur kurz (17 Tage) und liessen keine deutlichen Giftwirkungen erkennen. In einer 260 g schweren Ratte zeigte sich nach 11tägiger Einwirkung von intravenös injizierten 18 Urease-Einheiten nur eine schwache Bildung von Antiurease.

Sumner (l.c.) injizierte Kaninchen zur Darstellung von Antiurease mit wesentlich grösseren Mengen von Urease-Einheiten und zwar intraperitoneal während etwa 2 Monaten. Die Immunisierung konnte dann so stark werden, dass die Kaninchen an einem Tag über 1000 Urease-Einheiten ertrugen.

Zur Bestimmung der Antiurease empfielt sich folgendes Verfahren: Man setzt eine gewisse Menge Lösung krystall. Urease zu einer bestimmten Menge Antiurease-Lösung zu und inkubiert dann bei 37° $\frac{1}{2}$ Stunde, worauf die Urease-Antiurease-Fällung abzentrifugiert wird. In der übrig bleibenden Lösung wird die Urease in der üblichen Weise ermittelt.

Hinsichtlich der Chemie und Biochemie der Urease seien unter den bis jetzt vorliegenden Versuchsresultaten betont:

1) Urease ist auf die Harnstoff-Spaltung spezifisch eingestellt; wir fanden keine synthetischen Wirkungen, auch nicht auf die Systeme Ammoniak + Brenztraubensäure oder Ketoglutarsäure (H. Hasselquist).

2) Die Urease, Mol.Gew., gemessen durch Ultrazentrifuge, beträgt über 400 000 (J. B. Sumner, N. Gralen u. I. Eriksson-Quensel, J. Biol. Chem. *125*, 37 (1938). — R. B. Setlow, Arch. Biochem. Biophysics *36*, 328 (1952)). Die Urease ist thermolabil und in dieser Hinsicht stark pH-abhängig. Maximum der Stabilität pH 5,2–5,6. Isoelektrischer Punkt ~pH 5.

3) Urease wird enzymatisch (Papain und Pepsin) und in nicht-enzymatischer Phosphatlösung zu Spaltprodukten hydrolysiert, die durch Dialyse oder chromatographisch abgeschieden werden können.

Es kann als feststehend betrachtet werden, dass das Protein Urease als Antigen den Antikörper Antiurease hervorruft.

2. Antiasparaginase

In Zusammenhang mit unseren ersten Versuchen über Urease wurde in diesem Institut mit K. Brandting und B. Söderling versucht, die Bildung einer Antiasparaginase nachzuweisen.

Zur Darstellung der Asparaginase versetzten wir fein verteilte frische Kalbsleber mit 20 % Glycerin und pressten nach 6 Stunden den Brei stark aus. Vom dem so erhaltenen Pressaft wurden 10 Teile mit 1 Teil einer Mischung von 50 % Aceton, 45 % Isopropylalkohol und 5 % Essigsäure versetzt. Dadurch entstand eine (enzymatisch wenig aktive) Fällung, die durch scharfes Abzentrifugieren entfernt wurde. Der an Asparaginase angereicherte Rest wurde nun mit einer kalten wässrigen Phosphatpufferlösung pH 7,6 versetzt, dann in eine Kaolin-Suspension eingetragen und mit dieser bei 5–10° über Nacht gerührt. Die Elution von Kaolin erfolgte — entsprechend einem von R. Willstätter und E. Waldschmidt-Leitz zur Trypsin-Reinigung angewandten Verfahren — mit Ammoniumphosphat. Die erhaltene, mit Essigsäure auf pH 7,4 eingestellte Elution wurde nun im Vakuum auf $\frac{1}{5}$ des Volumens eingeengt und erwies sich gegenüber L Asparagin sehr wirksam (17 Asp.-Einh.).

1 Asparaginase-Einheit ist diejenige Enzymmenge, die 0,5 mMol Asparagin bei pH 8 im Volumen von 10 ml in 2 Stunden zur Hälfte spaltet.

Zu Immunitäts-Bestimmungen wurden zunächst Ratten verwendet, die aber schon in der zweiten Woche Vergiftungserscheinungen zeigten und nach intraperitonealer Injektion von ca. 3 Asp.-Einheiten verendeten. Wir wählten dann Kaninchen als Versuchstiere (Sven Gard; Marg. Rydbom; Sv. Steffenburg). Im Verlauf von 4 Wochen zeigte sich eine schwache aber *deutliche Immunisierung:* Von 6 Versuchstiere ertrugen 3 die intravenöse Injektion von 140 Asp.-Einheiten.

Bei orientierenden Versuchen über den (geringen) Einfluss der Acidität auf die Fähigkeit der Asparaginase Immunität zu erzeugen, wurde eine bemerkenswerte Beobachtung gemacht:

In 3 Kaninchen wurde unmittelbar nach den ersten 4 Asparaginase-Injektionen (zu je 10 Asp.-Einheiten) je 5 mg Desoxyribo-Nukleinsäure (DNS) subkutan injiziert, im ganzen 20 mg DNS. In allen 3 Kaninchen stieg die Entwicklung der Immunität durch die DNS-Zufuhr stark, so dass alle 3 Tiere bereits nach 3 Wochen eine intravenöse Injektion von wenigstens 220 Asp.-Einheiten ertrugen.

Der gleiche Effekt der DNS wurde weder bei einem entsprechenden Versuch mit unserem Urease-Präparate noch bei einem einzelnen Versuch mit Amino-polypeptidase (siehe unten) beobachtet.

Weitere Versuche, Immunitäts-Wirkungen an Antienzymen bzw. an Enzym-Antikörpern nachzuweisen haben wir an einer *Amino-polypeptidase* aus Darmschleimhaut von Kalb vorgenommen, und zwar nach einer von Waldschmidt-Leitz und Schäffner [10] beschriebenen Methodik.

Der Umstand, dass wir mit diesem Material nur unsichere Ergebnisse erhalten konnten, hängt vermutlich damit zusammen, dass in der Darmschleimhaut zahlreiche Enzyme vorkommen, die eine Reinigung der einzelnen Komponenten und eine Isolierung der Antienzyme erschweren.

Aus unseren Ergebnissen sowie aus den Angaben von Sumner geht hervor, dass die Antiurease als Immunität erzeugendes Antienzym, als Antigen, fungiert. Da man nunmehr fast alle Enzyme als Proteine ansehen darf, kann man von den Enzymen allgemein die Fähigkeit zur Bildung serologisch nachweisbarer Antikörper erwarten.

Unbeantwortet bleibt die Frage, ob und in welchem Grad hierbei Spezifität vorliegt.

In der neueren Literatur werden vielfach Inhibitoren enzymatischer Reaktionen den Antienzymen zugezählt. Offenbar handelt es sich hier um 2 Gruppen von Vorgängen, die in prinzipiell verschiedener Weise in den Stoffwechsel eingreifen.

Von den Enzym-Inhibitoren unterscheiden sich die in folgender Tabelle aufgenommenen Antienzyme besonders durch ihre Fähigkeit spezifische Immunität (in Kaninchen) bzw. Antisera hervorzurufen [11].

Antienzyme von	Literatur
Amylase	LÜERS u. ALBRECHT, Fermentforschung *8*, 52 (1924).
Lactase	SCHÜTZE, Z. Hyg. Infektionskrankh. *48*, 457 (1904).
Urease	SUMNER u. KIRK, Science *74*, 102 (1931).
Asparaginase	von EULER u. GARD, Experientia *5* (1948).
Pepsin	NORTHROP, J. Gen. Physiol. *13*, 789 (1930).
Pepsin	SACHS, Fortschr. Med. *29* (1902).
Chymotrypsin	TEN BROECK, J. Biol. Chem. *106*, 729 (1934).
Chymosin	J. MORGENROTH, Zentr. Bakteriol. Parasitenk. *26* u. *27* (1899–1900).
Thrombin	J. BORDET u. GENGOU, Ann. inst. Pasteur *15*, 720 (1901).

Als Antikatalase haben Batelli und Stern (1905) einen Hemmungsstoff der Hydroperoxyd-Spaltung bezeichnet, der wie $FeSO_4$ funktioniert haben dürfte.

R. Blix [12] hat gezeigt, dass die Aktivität von Katalase-Lösungen durch kurzes Erwärmen auf 60° stark erhöht wird. Für die Deutung, dass die Aktivierungs-Erhöhung durch die Inaktivierung oder Zerstörung einer Anti-Katalase verursacht wird, liegen keinerlei Anhaltspunkte vor.

Auffallend ist, dass bisher noch keine Immunisation durch die weit verbreiteten Dehydrogenasen nachgewiesen werden konnte.

Antivitamine

Nach Domagks Entdeckung der therapeutischen Wirkungen der Sulfonamide ist der Mechanismus dieser Wirkungen eingehend bearbeitet worden. Die Wachstumshemmung, welche Sulfanilsäure an gewissen Bakterien hervorbringt wird bekanntlich durch *p*-Aminobenzoesäure aufgehoben. Woods [13] und Fildes [13] ist es gelungen diesen Antagonismus aufzuklären. Es war nach Kuhn [14] „am einfachsten anzunehmen, dass es in den Bakterien ein spezifisches Protein oder einen Rezeptor gibt, der in umkehrbarer Weise sowohl mit der Carbonsäure als auch mit der Sulfonsäure sich vereinigt".

Es kann aber auch in Betracht gezogen werden, dass die *p*-Aminobenzoesäure nicht als solche wirksam ist, sondern erst nachdem sie zu einem neuen, als Coenzym fungierenden Molekül aufgebaut worden ist. In diesem Fall könnte die „Anti-Vitamin-Wirkung" auch darin bestehen, dass der Hemmungsstoff die *p*-Aminobenzoesäure von demjenigen Enzymsystem verdrängt, welches den Aufbau zu Coenzym bewirkt.

In diesem Sinn wurde in diesem Institut die „Anti-Vitamin-Wirkung" bearbeitet (15). Die wesentlichen Ergebnisse dieser Untersuchungen waren die folgenden:

1) Cozymasebedingte Dehydrasen (Glucose- und Lactico-Dehydrase) werden durch eine Reihe zyklischer Carbon- und Sulfonsäuren, darunter Salizylsäure, Nikotinsäure und Pyridin-β-sulfonsäure, sowie durch Adenin, Adenosin und Adeninnucleotide gehemmt.

2) Die durch Vertreter aus der Gruppe der zyklischen Säuren erzeugte Hemmung

kann durch Cozymase zurückgedrängt werden, die durch Vertreter der Adeningruppe hervorgerufene nicht.

3) Die unter 1) benannten Substanzen hemmen auch die Succinodehydrase.

4) Die Hemmungen werden weder bei der Lactico-, noch bei der Succinodehydrase durch höhere Konzentrationen des Substrates rückgängig gemacht.

Auf *Hormon*-Verdrängungen kommen wir an anderer Stelle zurück.

Stockholm, Universität, Vitamin-Institut.

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Studies on yeast invertase

XI. Inhibition by organic bases[1]

1. Aniline

By Karl Myrbäck

With 5 figures in the text

Inhibition of yeast invertase by aniline and certain aniline derivatives was first described by von Euler and Svanberg [1]. The inhibition by aniline was shown to occur immediately on mixing of enzyme and inhibitor; incubation did not increase the inhibition. Variation of the substrate concentration had no influence, i.e., the inhibition is non-competitive. The inhibition could be partly reversed by the addition of certain substances that react with aniline, e.g. benzaldehyde. The degree of inhibition by aniline was found to depend on the total amine concentration in a manner intimating an amine-enzyme equilbrium governed by the mass action law.

From a few experiments at different pH values the (erroneous) conclusion was drawn that in the range of optimal activity of the enzyme the inhibition by aromatic amines is independent of pH.

A number of aniline derivatives were tested as inhibitors at pH ~ 4.5. *p*-Toluidine, *p*-anisidine and aniline were found to have the most powerful action; *o*-aminobenzoic acid, on the other hand, was practically ineffective as an inhibitor. The same was found for diphenylamine and alanine.

As a tentative explanation for the inhibition it was assumed that the aromatic amines react with an aldehyde group of the enzyme to yield Schiff bases. The authors leave the question open, if the "aldehyde group" is involved in the binding of the substrate, which seems strange, since it had been found that the inhibition is non-competitive.

von Euler and Myrbäck [2] showed in experiments with *p*-toluidine that the inhibition is strongly dependent on pH; a concentration of *p*-toluidine that gives, e.g., 90 % inhibition at pH 5 has practically no action at all at pH 3. It was tentatively assumed that the free base and not the corresponding cation is the inhibitor (cf. Rona and Bloch [3]).

It was also found that two invertase preparations, having the "time values" 17 and 0.52, respectively, were inhibited to the same degree by a given concentration of amine. This is understandable since the concentrations of amine required are so high that the amounts bound by the enzyme (and possibly by impurities in the enzyme preparations) are negligible.

[1] Part X, *Arkiv Kemi 16*, 221 (1960).

Myrbäck [4, 5] investigated the inhibition of invertase by several aniline derivatives and found that if the relative activity of the enzyme is plotted versus the concentration of free base in the solutions, the inhibition appears to be rather independent of pH. The inhibiting power of monosubstituted aminobenzenes was found to increase with increasing basicity of the amine, but the position of the substituent in the benzene nucleus had also a marked influence, the *meta* derivatives being generally stronger inhibitors than *ortho* or *para* derivatives. The inhibitions were found to be completely reversible and non-competitive. This means that the group of the enzyme reacting with the amines is not involved in the binding of the substrate. Assuming that only the free bases react with the enzyme, apparent dissociation constants of some invertase–amine complexes were calculated.

In a later publication [6] it was pointed out that the formation of Schiff bases is not the only conceivable type of reaction of the enzyme with aromatic amines, and it was shown that assumption of a salt-like compound between the amine cation and the negatively charged enzyme molecule would also appear to give a resonable explanation of the experimental results on the dependence of the amine inhibition upon pH, i.e. if it be assumed that the amine cation reacts with the "acidic group" of the enzyme with $K_A = 1.6 \times 10^{-7}$ (see further below).

In the present paper new experiments with aniline are reported and the nature of the interaction of invertase and aniline is discussed.

Experimental

Enzyme. Several partially purified preparations from brewer's yeast were used, in most cases a preparation called B 18. This was obtained by autolysis of brewer's yeast, fractional ethanol precipitation, adsorption on aluminium hydroxide, elution with 0.0125 *M* phosphate buffer, pH 6.8, dialysis for 3 days (water changed at least three times a day). Dry weight 0.39 mg/ml. 1 ml enzyme solution hydrolyzed 100 ml 4.75 % sucrose solution to about 3.8 % in 5 min at pH 4.5 and 30°C. $If_{30} = 40.5$. The preparation contained 3.8 % nitrogen and about 80 % polysaccharide (anthrone reaction with mannose as standard).

Activity determination. Activity was determined as described earlier [7]. Volume in all experiments 100 ml, temperature 30°C. Redistilled water was used throughout. The relative activity, RA, is calculated on the activity without inhibitor *at the pH optimum* (~ 4.5). Substrate concentration, $[S] = 0.139$ *M*.

Regulation of pH. Desired pH values of the assay mixtures were obtained in three different ways, using

(1) sodium acetate–acetic acid buffer, containing 1 *M* sodium acetate. Usually 10 ml was used in each experiment giving a final sodium acetate concentration of 0.1 *M*. Variation of the acetate buffer concentration did not markedly influence the RA values.

(2) phosphate buffer, final concentration 0.05 *M*.

(3) diluted hydrochloric acid.

pH was determined in all assay mixtures by means of a glass electrode and an Universal pH meter 22, Radiometer, Copenhagen.

Amines. Commercial preparations were redistilled or recrystallized. Only freshly made aqueous solutions were used.

Preliminary experiments

Several series of experiments were carried out in order to verify certain results from earlier investigations.

Table 1. Reversibility of the inhibition by aniline.

Incubated at pH	Activity determination at pH	Relative activity
4.0	4.0	0.70
4.0	7.6	0.14
7.6	7.6	0.15
7.6	4.0	0.71

1. The inhibition by all aromatic amines tested is instantaneous; incubation of enzyme and amine before the activity determination does not result in an increased inhibition. This is valid at least for the pH range 4–8. Incubations were carried out at 30°C.

2. The amine inhibitions are perfectly reversible. The following series of experiments may be quoted:

Four incubation mixtures were prepared; they contained 1 ml invertase solution, 1 ml acetate buffer, 10 ml aniline solution, 5.5×10^{-3} *M*, and water to 50 ml. In two of the mixtures pH was 4.0, in the other two 7.6. After 30 min at 30°, 50 ml substrate–buffer solution was added in each experiment. These solutions contained each 10 ml acetate buffer, calculated to give a final pH of either 4.0 or 7.6. The results of the activity determinations (Table 1) show the complete reversibility of the amine inhibition.

3. The non-competitive nature of the inhibition was verified for several aromatic amines in the pH range $3.7 - 7.7$. This means that, at least in this range, the amines interact with one or more groups of the enzyme that are not involved in the binding of the substrate. This fact simplifies the mathematical treatment of the data considerably, since the enzyme–substrate equilibrium can be disregarded altogether.

It should be noted that in the pH range mentioned the activity pH curve of yeast invertase (i.e., in the absence of any inhibitor) is independent of [S] [8]; the group with $pK_A \sim 6.8$ is not involved in the binding of the substrate. The *acid branch* of the activity pH curve, on the other hand, is dependent on substrate concentration ("competitive inhibition by H^+") [9]. However, as will be shown in the following, experiments on the amine inhibition at pH values below ~ 4 are of little interest for the theoretical explanation of the enzyme–amine interaction, and experiments at such pH values were therefore carried out only exceptionally.

5. In the following calculations K_A is the "dissociation constant of the weakly acidic group of invertase" (Michaelis). The values for K_A, given by different investigators vary somewhat; usually they are in the range $10^{-7} - 3 \times 10^{-7}$. The observed differences may be, at least partly, caused by variations of the buffer concentration and the type of buffer. Since in the majority of the experiments reported here acetate buffer, containing 0.1 *M* sodium acetate, was used, the activity pH curve was redetermined with this buffer. The average value of K_A, as determined in experiments with several invertase preparations was 1.6×10^{-7}, and this value has been used in the calculations.

The activity pH curve

The enzyme is regarded as an ampholyte, provisionally designated as $HENH_2$; the isoelectric point is assumed to be in the vicinity of 5. In less acid solution the ampholyte gives off a proton with $K_A = 1.6 \times 10^{-7}$ to yield the anion $^-ENH_2$. In more acid solution a proton is added with $K_X \sim 10^{-3}$ to yield the cation $HENH_3^+$. Since the activity pH curve is independent of substrate concentration above pH ~ 4.5 but dependent on substrate concentration in more acid solution, it is concluded [5] that $HENH_2$ and $^-ENH_2$, but not $HENH_3^+$, bind the substrate to yield $HENH_2S$ and $^-ENH_2S$. $HENH_2S$ is the only active species.

If K_M is the Michaelis constant (regarded as the dissociation constant of the enzyme–substrate compound), Σ = total enzyme concentration, and [S] the substrate concentration, we have

$$([HENH_2] + [^-ENH_2])\,[S] = K_M\,([HENH_2S] + [^-ENH_2S])$$

$$[H^+]\,([HENH_2] + [^-ENH_2]) = K_X\,([HENH_3^+] + [^-ENH_3^+])$$

$$[H^+]\,([^-ENH_2S] + [^-ENH_2] + [^-ENH_3^+]) = K_A\,([HENH_2S] + [HENH_2] + [HENH_3^-])$$

$$\Sigma = [HENH_2S] + [^-ENH_2S] + [HENH_2] + [^-ENH_2] + [HENH_3^+] + [^-ENH_3^+]$$

Elimination gives

$$\text{Relative activity} = \frac{[HENH_2S]}{\Sigma} = \frac{1}{\left(1 + \frac{K_A}{[H^+]}\right)\left[1 + \frac{K_M}{[S]}\left(1 + \frac{[H^+]}{K_X}\right)\right]} \qquad (1)$$

In the derivation of this formula it has been assumed that the acid branch of the activity pH curve, like the alkaline branch, has the form of a simple dissociation curve. The precise form of the acid branch is difficult to establish in consequence of the instability of yeast invertase in acid solution below pH ~ 3. It would appear, however, from curves determined by several authors, that in the acid region the activity decreases more rapidly than expected. If that is so, an explanation can be found under the plausible assumption that for the binding of the substrate more than one basic group is required [10]. If we assume that n such groups with approximately the same K_X value are involved, formula (1) changes to

$$\text{Relative activity} = \frac{1}{\left(1 + \frac{K_A}{[H^+]}\right)\left[1 + \frac{K_M}{[S]}\left(1 + \frac{[H^+]}{K_X}\right)^n\right]} \qquad (2)$$

It is easily seen that above pH ~ 4.5 formulas (1) and (2) give the same result; since $K_X = 10^{-3}$ or greater, $[H^+]/K_X$ will be negligible. The formula for the *alkaline branch* of the activity pH curve can be written

$$\text{Relative activity} = \frac{1}{\left(1 + \frac{K_A}{[H^+]}\right)\left(1 + \frac{K_M}{[S]}\right)} \qquad (3)$$

If in a series of experiments [S] is kept constant and pH is varied between 4.5 and 8, and if the activity *at this substrate concentration* and pH = 4.5 is made equal to 1, then the apparent relative activity in the pH range mentioned is

$$\mathrm{RA} = \frac{1}{1 + K_A/[H^+]} \tag{4}$$

In the presence of certain inhibitors not only a general depression of the enzymatic activity is observed, but the form of the activity pH curve may be very strongly altered. Several heterocyclic bases, e.g. pyridine, inhibit invertase, and the inhibition is characteristically dependent on pH; it increases towards the alkaline region more or less strongly dependent on the base strength of the inhibitor. The concentrations of the bases, necessary for marked inhibition, are high compared to the molecular concentration of the enzyme, and the amounts of base bound by the enzyme are negligible. If it be assumed that the cation BH^+ of the base B (total concentration [B]) reacts with the enzyme anion to form an inactive, salt-like compound, we have in an enzyme–substrate–inhibitor solution, beside the equilibria stated above, also

$$[BH^+]([^-ENH_2S] + [^-ENH_2] + [^-ENH_3^-]) = K_Y([BENH_2S] + [BENH_2] + [BENH_3^+])$$

where K_Y is the dissociation constant of the enzyme-inhibitor compound. Elimination gives

$$\text{Relative activity} = \frac{1}{\left[1 + \frac{K_M}{[S]}\left(1 + \frac{[H^+]}{K_X}\right)\right]\left[1 + \frac{K_A}{[H^+]}\left(1 + \frac{[B]}{K_Y} \cdot \frac{[H^+]}{[H^+] + K_B}\right)\right]} \tag{5}$$

where K_B is the ionization constant of the base B. Working with a constant [S] and in the pH range 4.5–8, this formula can be simplified as shown above for formulas (1) and (2); cf. formula (10).

Dependence of the aniline inhibition on the inhibitor concentration

Series of experiments were carried out with various concentrations of aniline; in each series the pH was kept constant. Since the total concentration of aniline, $[An_{tot}]$, is $> 10^{-5}$ M, it is evident that the amounts of aniline bound by the enzyme are negligible compared to $[An_{tot}]$. At a given pH the concentration, [An], of free aniline base, and the concentration, $[AnH^+]$, of the anilinium ion are proportional to the total aniline concentration. Irrespective of whether the free base or det anilinium ion or both react with the enzyme, we can use $[An_{tot}]$ to determine the dependence of the inhibition on the inhibitor concentration. If we assume that n inhibitor molecules must be bound by one enzyme molecule in order to render it inactive, we have

$$K = \frac{[\text{free enzyme}]\,[An_{tot}]^n}{[\text{bound enzyme}]} \tag{6}$$

where K is the *apparent* dissociation constant of the enzyme–aniline compound.

Table 2. Dependence of the inhibition upon aniline concentration.

$\log [An_{tot}]$	RA found	$\log \frac{1-RA}{RA}$	$\log [An_{tot}]$	RA found	$\log \frac{1-RA}{RA}$
	pH 4.15			pH 5.3	
−4.00	0.90	−0.97	−3.26	0.52	−0.04
−3.70	0.80	−0.60	−2.96	0.35	+0.19
−3.22	0.60	−0.18	−2.78	0.30	+0.37
−2.70	0.33	+0.30	−2.78	0.30	+0.37
−2.40	0.19	+0.63			
−2.10	0.10	+0.95			
	pH 5.45			pH unknown[1]	
−3.96	0.80	−0.60	−3.75	0.63	−0.23
−3.66	0.69	−0.34	−5.35	0.38	+0.21
−3.36	0.51	−0.01	−3.05	0.24	+0.50
−2.96	0.29	+0.40	−2.75	0.165	+0.70
−2.66	0.21	+0.57	−1.75	0.015	+1.82
−2.36	0.10	+0.95			

[1] Values taken from [1], p. 35; the pH is not stated explicitly.

Since $$\frac{[\text{free enzyme}]}{[\text{bound enzyme}]} = \frac{RA}{1-RA}$$

we have $$[An_{tot}]^n = K\frac{1-RA}{RA}$$

or $$n \cdot \log [An_{tot}] = \log K + \log \frac{1-RA}{RA} \qquad (7)$$

Experiments with several concentrations of aniline were carried out to test the validity of formula (7) and to determine the value of n (Table 2).

In Fig. 1 $\log (1-RA)/RA$ has been plotted versus $\log [An_{tot}]$. It is seen that for each pH value the experimental points fall on straight lines making 45° angles with the abscissa. This means that n in formula (7) = 1, i.e. one enzyme molecule is inactivated by the reaction with one aniline molecule.

The *apparent* dissociation constants are found as the points of intersection with the abscissa; these constants are, however, of little interest. Fig. 1 shows that the apparent constants decrease (i.e., the inhibition increases) with increasing pH. As mentioned before, this has led to the belief that the inhibitor is the free amine base whose concentration for a constant value of $[An_{tot}]$ increases with increasing pH. As will be shown in the following, this is not the only possible explanation.

Theoretical

Since the inhibition of yeast invertase by aromatic amines is non-competitive, we may disregard the enzyme-substrate equilibrium. Inhibition experiments with amines in the pH region below ~4 are of little interest (see below); therefore we disregard the "dissociation of the basic groups of the enzyme" (Michaelis) which has a marked influence on the enzyme activity only below pH ~4. The following simplified notations are used:

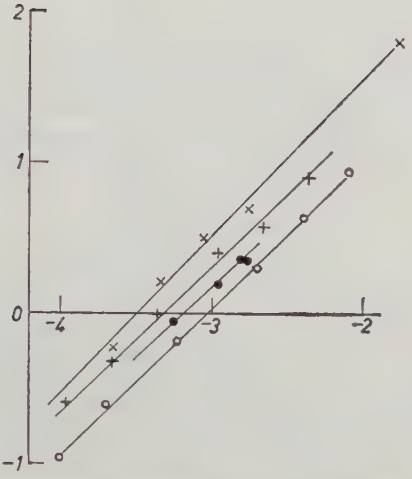

Fig. 1. Dependence of the relative activity upon the inhibitor concentration. Abscissa, log $[An_{tot}]$; ordinate, log $(1-RA)/RA$.
○, pH = 4.15, ●, pH = 5.30; +, pH = 5.45; ×, pH unknown [1].

RA, the relative activity referred to the activity without inhibitor *at the pH optimum* and the substrate concentration (0.139 *M*) used in all experiments.

HE, the active form of the enzyme ("the undissociated weak acid" according to Michaelis)

$K_A = [H^+][E^-]/[HE] = 1.6 \times 10^{-7}$, the "dissociation constant of the weak acid".

$[An_{tot}]$, total aniline concentration.

[An], concentration of free amine base.

$[AnH^+]$, concentration of the anilinium ion.

$K_B = [H^+][An]/[AnH^+]$, the base constant of aniline $= 2.5 \times 10^{-5}$.

[AnE], the concentration of the invertase–aniline compound.

1. The free amine base reacts with the enzyme

(*a*) We assume that the free amine base reacts with a group in the enzyme molecule, e.g., an aldehyde group, that does not alter its charge in the pH region in question (pH 4–8). This means that E^- as well as HE react with the amine. If Σ is the total enzyme concentration, we have

$$[H^+][E^-] = K_A[HE]$$
$$([HE] + [E^-])[An] = K_1([AnHE] + [AnE])$$
$$\Sigma = [HE] + [E^-] + [AnHE] + [AnE].$$

Elimination gives

$$\text{Relative activity} = \frac{[HE]}{\Sigma} = \frac{1}{\left(1 + \frac{K_A}{[H^+]}\right)\left(1 + \frac{[An_{tot}]}{K_1} \cdot \frac{K_B}{K_B + [H^+]}\right)} \qquad (8)$$

where K_1 is the dissociation constant of the enzyme–amine compound.

(*b*) We assume that the free amine base reacts with, e.g., an aldehyde group in the *isoelectric* form of the enzyme (HE) but not in the enzyme anion (E^-). Admittedly this assumption does not appear very probable; it is difficult to see why an aldehyde group should react with the amine only when the so-called acidic group of the enzyme is protonated. However, on this assumption we have

$$[H^+][E^-] = K_A[HE]$$
$$[HE][An] = K_2[AnHE]$$
$$\Sigma = [HE] + [E^-] + [AnHE]$$

and

$$RA = \frac{[HE]}{\Sigma} = \frac{1}{1 + \frac{K_A}{[H^+]} + \frac{[An_{tot}]}{K_2} \cdot \frac{K_B}{K_B + [H^+]}} \tag{9}$$

where K_2 is the dissociation constant of the enzyme–amine compound.

2. The amine cation reacts with the enzyme

As mentioned before, several heterocyclic bases which cannot be supposed to react with aldehyde groups are fairly strong inhibitors. In this case the explanation would be that the cations of the bases react with the enzyme anion. It appears necessary, therefore, to examine also the inhibition by the aromatic amines from this point of view.

$$[H^+][E^-] = K_A[HE]$$
$$[AnH^+][E^-] = K_3[AnE]$$
$$\Sigma = [HE] + [E^-] + [AnE]$$

and

$$RA = \frac{[HE]}{\Sigma} = \frac{1}{1 + \frac{K_A}{[H^+]} + \frac{[An_{tot}]}{K_3} \cdot \frac{K_A}{K_B + [H^+]}} \tag{10}$$

where K_3 is the dissociation constant of the enzyme–amine compound.

Comparing the formulas (9) and (10) it is seen that if

$$K_B/K_2 = K_A/K_3$$

the formulas give identical results. This will necessarily be the case if K_2 and K_3 are calculated by insertion of experimental values of RA for a certain pH in the respective formulas. In other words, the inhibition experiments at various pH values do not allow us to distinguish between the assumptions *1 (b)* and *2*.

Since $K_A = 1.6 \times 10^{-7}$, we find that for $[H^+] > 10^{-4}$, $K_A/[H^+]$ is negligible, and even at pH = 5 the factor $(1 + K_A/[H^+])$ is as small as 1.016. Thus in the region below pH = 4 and even below pH = 5, the formulas (8) and (10) can be simplified to

$$RA = \frac{1}{1 + [An_{tot}] \frac{K_B}{K_1(K_B + [H^+])}} \tag{8a}$$

and

$$RA = \frac{1}{1 + [An_{tot}] \dfrac{K_A}{K_3 (K_B + [H^+])}} \qquad (10\,a)$$

It is seen from these formulas that in the pH region below ~ 5 they will give practically identical results provided K_1 and K_3 are selected to satisfy the equation

$$K_3 = K_1 K_A / K_b \qquad (11)$$

This will be the case if RA values for pH = 4–5 are used for the calculation of K_1 and K_3. Experiments on the inhibition in the pH region below 5 can therefore not be used to decide if the interaction between yeast invertase and aniline (and other primary aromatic amines) is the formation of a Schiff base (mechanism *1*) or the formation of a salt-like compound (mechanism *2*). However, even if the constants satisfy equation (11), formulas (8) and (10) yield different results in the pH region above ~ 5. Therefore, it should be possible from inhibition experiments in the pH region 5–8 to decide between, on the one hand, mechanism *1 (a)*, and on the other, mechanisms *1 (b)* and *2*.

Inhibition of invertase by aniline and its dependence on pH

(*a*) $[An_{tot}] = 5.5 \times 10^{-4}\ M$

The RA in the presence of $5.5 \times 10^{-4}\ M$ aniline was determined at various pH values (Table 3). From the experimental value RA = 0.52 for pH = 5.0 and using the values $K_A = 1.6 \times 10^{-7}$ and $K_B = 2.5 \times 10^{-5}$, we calculate

$$K_1 = 4.4 \times 10^{-4} \text{ (equation (8))},$$

and

$$K_3 = 2.8 \times 10^{-6} \text{ (equation (10))}.$$

From these values, RA was calculated for various pH values according to formulas (8) and (10), respectively (Table 3). The inhibition pH curve is shown in Fig. 2. Table 3 and Fig. 2 show that the RA values calculated according to formula (10) are in a considerably better agreement with the experimental values than the values calculated from formula (8). It would appear then that the assumption of a salt-like compound between enzyme and the base cation better explains the experiments than the assumption of the formation of a Schiff base. As mentioned before, the experiments do not allow a distinction between mechanisms *1 (a)* and *2*, but the fact that certain heterocyclic bases act as inhibitors would seem to exclude mechanism *1 (b)*.

(*b*) $[An_{tot}] = 1.10 \times 10^{-3}\ M$

RA values in the presence of $1.10 \times 10^{-3}\ M$ aniline were determined in the pH range 5–8 (Table 4). Using the values $K_1 = 4.4 \times 10^{-4}$ and $K_3 = 2.8 \times 10^{-6}$, RA was calculated according to formulas (8) and (10), respectively. Table 4 and Fig. 3 show that the RA values determined in phosphate buffer and hydrochloric acid, respectively, agree very well. Furthermore it is obvious that the RA values calculated according to formula (10) agree well with the experimental values, whereas

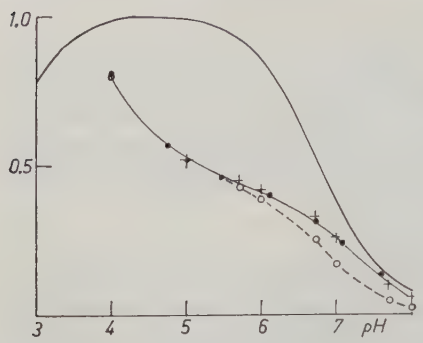

Fig. 2.

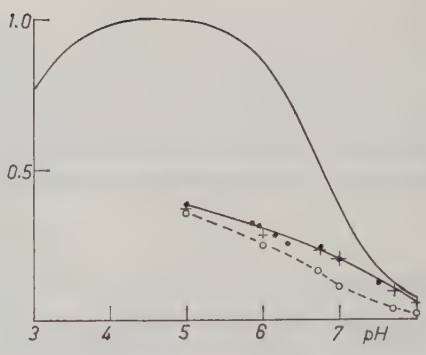

Fig. 3.

Fig. 2. Inhibition pH curves for $[An_{tot}] = 5.5 \times 10^{-4}$ *M*. Ordinate: Rel. activity. ●, experimental values; ○, values calculated from formula (8); +, values calculated from formula (10).

Fig. 3. Inhibition pH curves for $[An_{tot}] = 1.10 \times 10^{-3}$ *M*. ●, experimental values; ○, values calculated from formula (8); +, values calculated from formula (10).

Table 3. Relative activity for $[An_{tot}] = 5.5 \times 10^{-4}$ *M* and various pH values. Acetate buffer.

Experimental		Calculated		
pH	RA	pH	formula (8)	formula (10)
4.00	0.81	4.00	0.80	0.80
4.75	0.57	5.00	0.52	0.52
5.00	0.52	5.70	0.43	0.45
5.45	0.46	6.00	0.39	0.42
6.10	0.40	6.70	0.25	0.33
6.70	0.31	7.00	0.17	0.26
7.05	0.24	7.70	0.05	0.10
7.60	0.14	8.00	0.03	0.06

those calculated from formula (8) deviate markedly. It should be noted that the calculations are based on values of the constants K_1 and K_3, determined with 5.5×10^{-4} *M* aniline and acetate buffer.

If we disregard the somewhat improbable possibility that the free amine base should react only with the isoelectric enzyme to form a Schiff base (mechanism *1 (b)*), the experiments support the assumption that inhibition of yeast invertase by aniline is caused by an interaction of the anilinium ion with the negatively charged enzyme ion. Admittedly the difference between the values calculated from equation (8) and (10), respectively, are not *very* great, but they show a regularity in the dependence on pH that can hardly be a coincidence.

Although the assumption of a reaction between the anilinium ion and the enzyme anion gives a very good agreement between experiments and calculated values, it should be admitted that the choice of the K_A value of the enzyme

Table 4. Relative activity for $[An_{tot}] = 1.10 \times 10^{-3}$ M and various pH values. Phosphate buffer or HCl.

Experimental pH	pH regulated with	RA	Calculated pH	formula (8)	formula (10)
5.00	Phosphate	0.38	5.00	0.35	0.36
5.00	HCl	0.38	6.00	0.25	0.28
5.85	Phosphate	0.32	6.70	0.16	0.23
5.95	HCl	0.31	7.00	0.11	0.20
6.15	Phosphate	0.28	7.70	0.03	0.09
6.30	Phosphate	0.25	8.00	0.02	0.05
6.75	Phosphate	0.24			
7.00	Phosphate	0.20			
7.50	Phosphate	0.12			

may appear arbitrary. Since the enzyme protein very probably contains quite a number of "acidic" groups of varying K_A values, it seems necessary to examine assumptions of a reaction of the amine cation with enzyme groups of other K_A values than 1.6×10^{-7}.

Let us assume that the amine cation reacts with a weakly acidic group with $K_{A1} < K_A$. If the isoelectric form of the enzyme is denoted by H_2E, we have the following equilibria

$$[H^+][E^{2-}] = K_{A1}[HE^-]$$
$$[H^+][HE^-] = K_A[H_2E]$$
$$[AnH^+][E^{2-}] = K_4[AnE]$$
$$\Sigma = [H_2E] + [HE^-] + [E^{2-}] + [AnE]$$

and

$$RA = \frac{[H_2E]}{\Sigma} = \frac{1}{1 + \frac{K_A}{[H^+]}\left(1 + \frac{K_{A1}}{[H^+]} + \frac{[An_{tot}]}{K_4} \cdot \frac{K_{A1}}{K_B + [H^+]}\right)} \qquad (12)$$

Let us assume that $K_{A1} = 10^{-8}$. Inserting this value and $RA = 0.52$ for $pH = 5$ in formula (12) we find $K_4 = 2.8 \times 10^{-9}$. With this value and the assumed value for K_{A1}, RA values for various pH values have been calculated. Table 5 and Fig. 4 show that the calculated inhibition pH-curve differs completely from the experimental curve, and it is easily seen from equation (12) that other values of of $K_{A1} < 1.6 \times 10^{-7}$ give practically the same curve as $K_{A1} = 10^{-8}$, if the value $RA = 0.52$ for $pH = 5$ is used for calculation of K_4. Consequently, the assumption of a reaction of the anilinium ion with an acidic group of the enzyme with $K_{A1} < K_A$ does not explain the experimental results.

A somewhat far-fetched assumption would be that the anilinium ion reacts with the group with $K_A = 1.6 \times 10^{-7}$ as well as with a more weakly acidic group with $K_{A1} < K_A$. This assumption leads to the following expression for RA

$$RA = \frac{1}{1 + \left(1 + \frac{K_{A1}}{[H^+]}\right)\left(\frac{K_A}{[H^+]} + \frac{[An_{tot}]}{K_5} \cdot \frac{K_A}{K_B + [H^+]}\right)} \qquad (13)$$

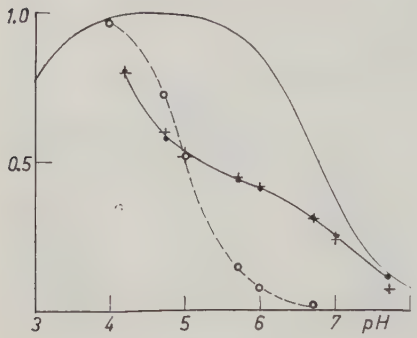

Fig. 4.

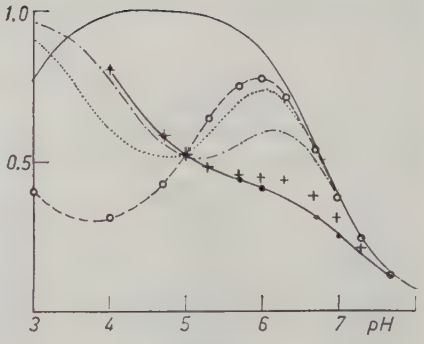

Fig. 5.

Fig. 4. Inhibition pH curves for $[An_{tot}] = 5.5 \times 10^{-4}$ *M*.
●, experimental values; ○, values calculated from formula (12); +, values calculated from formula (13).

Fig. 5. Inhibition pH curves for $[An_{tot}] = 5.5 \times 10^{-4}$ *M*.
—●, experimental values; – – –○, formula (14) and $K_{A2} = 10^{-3}$; ···, formula (14) and $K_{A2} = 2 \times 10^{-5}$, -·-·-, formula (14) and $K_{A2} = 2 \times 10^{-6}$; + + +, formula (14) and $K_{A2} = 1.6 \times^{-7}$.

Table 5. Relative activity for $[An_{tot}] = 5.5 \times 10^{-4}$ *M* aniline. The amine cation is assumed to react with a weakly acidic group with $K_{A1} < K_A$.

	Relative activity		
		Calculated	
pH	Experimental	Formula (12) $K_{A1} = 10^{-8}$	Formula (13) $K_{A1} = 10^{-8}$
4.00	0.81	0.97	0.80
4.70	0.58	0.73	0.60
5.00	0.52	(0.52)	(0.52)
5.70	0.44	0.15	0.45
6.00	0.41	0.08	0.42
6.70	0.31	0.02	0.31
7.00	0.25	0.01	0.24
7.70	0.12	—	0.07

If we insert in formula (13) the values $K_{A1} = 10^{-8}$, $K_A = 1.6 \times 10^{-7}$, RA = 0.52, and $[H^+] = 10^{-5}$, we find $K_5 = 2.8 \times 10^{-6}$. If this value is used to calculate RA for other pH values according to equation (13) we get the values recorded in Table 5. The agreement with the experimental values is very good. However, a comparison of formulas (10) and (13) shows that this is more or less self-evident ($K_{A1}/[H^+]$ is very small below pH = 7) and anyway, since the group with $K_A = 1.6 \times 10^{-7}$ is involved, the assumption made has no special interest.

Less improbable appears an assumption that the anilinium ion reacts with a group of the enzyme molecule having $K_{A2} > K_A$. Using the same notations as above, we have

Table 6. Relative activity for $[An_{tot}] = 5.5 \times 10^{-4}$ *M* aniline. The amine cation is assumed to react with an acidic group with $K_{A2} > K_A$.

K_{A2} ...		10^{-3}	2×10^{-4}	2×10^{-5}	2×10^{-6}	1.6×10^{-7}
K_6 ...		1.78×10^{-4}	1.69×10^{-4}	1.17×10^{-4}	2.89×10^{-5}	2.73×10^{-6}
pH	RA, exptl.	RA, calculated (equation (14))				
3.0	—	0.40	0.68	0.91	0.96	0.97
4.0	0.81	0.31	0.37	0.61	0.77	0.79
4.7	0.58	0.42	—	—	0.56	0.58
5.0	0.52	(0.52)	(0.52)	(0.52)	(0.52)	(0.52)
5.3	0.48	0.64	—	—	0.51	0.48
5.7	0.44	0.75	—	—	0.56	0.45
6.0	0.41	0.77	0.77	0.74	0.60	0.44
6.3	0.38	0.71	—	—	0.60	0.43
6.7	0.31	0.54	—	—	0.51	0.38
7.0	0.25	0.38	0.38	0.38	0.37	0.31
7.3	0.19	0.24	—	—	0.23	0.21
7.7	0.12	0.11	—	—	0.11	0.11

$$[H^+][E^{2-}] = K_A[HE^-]$$

$$[H^+][HE^-] = K_{A2}[H_2E]$$

$$[AnH^+]([HE^-] + [E^{2-}]) = K_6([HEAn] + [EAn])$$

In this case H_2E and HE^- are both enzymatically active.

$$\Sigma = [HE^-] + [H_2E] + [E^{2-}] + [HEAn] + [EAn]$$

$$RA = \frac{[HE^-]}{\Sigma}\left(1 + \frac{[H^+]}{K_{A2}}\right)$$

$$1 - RA = \frac{[HE^-]}{\Sigma}\left(\frac{K_A}{[H^+]} + \frac{[AnH^+]}{K_6} \cdot \frac{K_A + [H^+]}{[H^+]}\right)$$

Eliminating $[HE^-]/\Sigma$ we get

$$RA = \frac{1}{1 + \frac{K_{A2}}{K_{A2} + [H^+]}\left(\frac{K_A}{[H^+]} + \frac{[H^+] + K_A}{[H^+] + K_B} \cdot \frac{[An_{tot}]}{K_6}\right)} \qquad (14)$$

Assuming various values for K_{A1} and using the value $RA = 0.52$ for $[H^+] = 10^{-5}$, K_6 was calculated and used for calculation of theoretical inhibition pH curves (Table 6 and Fig. 5). Obviously the only assumption that gives a resonably good agreement with the experiments is $K_{A2} = K_A = 1.6 \times 10^{-7}$. It seems therefore that, if the inhibition by the aromatic amines is caused by a reaction between the amine cation and a negative enzyme group, this group is the "weakly acidic group", postulated by Michaelis as an explanation of the activity pH curve of yeast invertase.

SUMMARY

The inhibition of yeast invertase by aniline, and especially the dependence of the inhibition upon pH has been investigated. The inhibition is instantaneous, non-competitive and completely reversible.

In earlier investigations on the inhibition of yeast invertase by primary aromatic amines it was tentatively assumed that the free amine bases react with an aldehyde group of the enzyme forming Schiff bases. Since the enzyme is inhibited in a similar manner by several heterocyclic bases which cannot be supposed to form Schiff bases, other possible explanations for the mechanism of the inhibition have been examined.

On the assumption that the cations of the bases form salt-like compounds with the negatively charged invertase ion, a good agreement between calculated and experimental values is found. The assumption that the free amine bases react with a non-ionic group of the enzyme molecule, e. g. an aldehyde group, does not give the same good agreement. It must be admitted, however, that if the free amine bases should react with the so-called aldehyde group in the *uncharged* form of the enzyme exclusively and not with the same group in the negative enzyme ion, the calculations give results identical with those derived from the assumption of a reaction between the base cation and the enzyme anion.

Calculations based on various values of the ionization constant of the group of the enzyme reacting with the base cation appear to show that the only value which gives results in reasonably good agreement with the experiments is $K_A = 1.6 \times 10^{-7}$, i.e. the value postulated by Michaelis in order to explain the alkaline branch of the activity pH curve of yeast invertase.

The activity determinations have been carried out by Mrs Ebba Willstaedt whose assistance is gratefully acknowledged.

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Studies on yeast invertase

XII. Inhibition by organic bases[1]

2. Some heterocyclic bases

By Karl Myrbäck and Ebba Willstaedt

With 5 figures in the text

The inhibition of yeast invertase by aniline and certain other primary aromatic amines has been tentatively explained as due to a Schiff base formation between the amine and an aldehyde group of the enzyme molecule [1–4]. The inhibition increases with increasing pH in the range 3–8, i.e., the pH range where the enzyme activity can be accurately determined. Since the primary aromatic amines tested generally are comparatively weak bases with $pK_B = 3$–6, they are partly ionized in the pH range in question, and the dependence of the invertase inhibition on pH appears to show that either the uncharged amine bases or the corresponding cations, but not both, are the true inhibitors. It should not be forgotten, however, that the enzyme itself ionizes in the same pH range.

The assumption of a Schiff base formation as the mechanism of the inhibition obviously means that the free amine bases and not the corresponding cations are regarded as the inhibitory agents, and since the concentration of the free amine bases increases with increasing pH, the dependence of the inhibition on pH would appear understandable. If for a given aromatic amine the inhibition (relative activity, RA) is calculated, not on the total amine concentration, but on the concentration of free base, the inhibition is found to be rather independent of the pH [4].

Assuming the formation of a Schiff base between the free amine base and an aldehyde group of the enzyme, RA in the presence of an aromatic amine is calculated according to equation (8);[2] the relative activity is referred to the activity without inhibitor *at the* pH *optimum* (~ 4.5). If the concentration of free base is denoted as [B], equation (8) can be written

$$\text{Relative activity} = \frac{1}{\left(1+\frac{K_A}{[H^+]}\right)\left(1+\frac{[B]}{K_1}\right)}$$

Without inhibitor the relative activity, calculated on the activity at the pH optimum,

[1] Part XI (ref. [5]) appeared earlier in these archives (*17*, 213 (1960)).

[2] Equations (1)–(14) will be found in Part XI of this series [5].

is $1/(1 + K_A/[H^+])$ and, obviously, in the presence of a certain concentration, [B], of free amine base, the relative activity RA′, *calculated on the activity without inhibitor at the same pH*, will be $RA' = 1/(1 + [B]/K_1)$ i.e., the relative activity, calculated in this way, should be independent of the pH.

As will be shown in the present communication, yeast invertase is inhibited by certain heterocyclic bases which cannot be supposed to react with the enzyme through the formation of Schiff bases. The inhibition depends on pH in a manner similar to that of the inhibition by the primary aromatic amines. The only plausible explanation of the invertase inhibition by the heterocyclic bases seems to be that the positive ion of the base, BH^+, reacts with the negatively charged enzyme, E^-. On the assumption that BH^+ reacts with a group of the enzyme molecule with $K_A = 1.6 \times 10^{-7}$, the inhibition pH curve is represented by equation (5). At a constant substrate concentration the simplified equation (15) can be used; this equation is, of course, identical with equation (10) in [5].

$$RA = \frac{1}{1 + \frac{K_A}{[H^+]}\left(1 + \frac{[B_{tot}]}{K_3} \cdot \frac{[H^+]}{K_B + [H^+]}\right)} \qquad (15)$$

This equation can also be written

$$RA = \frac{1}{1 + \frac{K_A}{[H^+]}\left(1 + \frac{[BH^+]}{K_3}\right)}$$

It has been found [5] that in the case of the inhibition by aniline, RA values calculated according to equation (15) agree better with the experimental values than those calculated according to equation (8), i.e. on the assumption of the formation of a Schiff base.

Experimental

Enzyme. Partly purified preparations from brewer's yeast as described in Part XI [5].

Activity determination as described earlier [6].

Regulation of pH. pH of the assay mixtures was regulated by (*a*) hydrochloric acid, (*b*) acetate buffer (final concentration 0.1 *M* NaAc), or (*c*) phosphate buffer (final concentration 0.05 *M*). In the case of strong bases these were neutralized to pH ~ 5 with hydrochloric acid and an appropriate buffer added. pH was determined in the assay mixtures by means of a glass electrode and the Universal pH Meter 22, Radiometer, Copenhagen.

Bases. The preparations used were best quality commercial preparations. Only freshly made aqueous solutions were used.

Experiments with pyridine

(a) *Dependence of inhibition on inhibitor concentration*

A few experiments with acetate buffer and various concentrations of pyridine, $[Py_{tot}]$, were carried out; pH was 4.70 ± 0.03 (Table 1). In Fig. 1, $\log[(1 - RA)/RA]$ has been plotted versus $\log[Py_{tot}]$. The experimental points are on a straight line with slope 1. This means that 1 enzyme molecule is inactivated by 1 pyridine molecule. The same result has been found for aniline [5].

Table 1. Relative activity (RA) at various pyridine concentrations; pH = 4.70.

$\log [Py_{tot}]$	RA	$\log [(1-RA)/RA]$
−1.605	0.74	−0.54
−1.207	0.54	−0.07
−0.910	0.46	+0.07
−0.605	0.24	+0.50

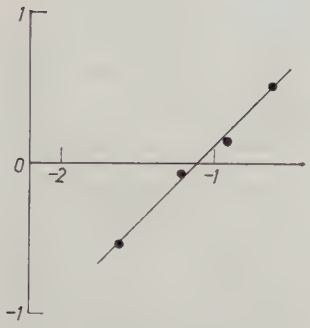

Fig. 1.

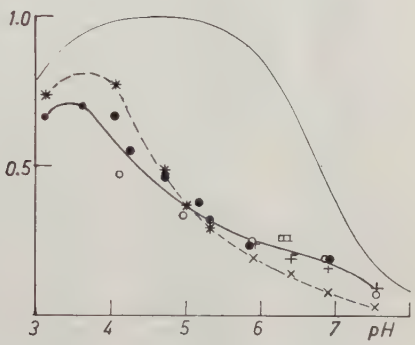

Fig. 2.

Fig. 1. Dependence of the relative activity upon the inhibitor concentration. Abscissa, log Py_{tot}; ordinate, log [(1 − RA)/RA].

Fig. 2. Inhibition pH curve for $Py_{tot} = 0.124$ *M*. ●, experiments with HCl; ○, experiments with acetate buffer; □, experiments with phosphate buffer; +, values calculated from equation (15); ×, values calculated from equation (8). Ordinate, relative activity.

(b) *Experiments with* $[Py_{tot}] = 0.124$ *M at various pH values*

Inhibition experiments were carried out using acetate or phosphate buffer or HCl for the regulation of pH. In a few experiments enzyme and pyridine were incubated together for 30 min at 30°C and the pH indicated. Table 2 shows that incubation of enzyme and inhibitor does not increase the inhibition, i.e., the formation of the enzyme-inhibitor compound is instantaneous.

Table 2 and Fig. 2 show that the inhibition experiments in acetate buffer and in HCl do not agree too well. However, if the continuous curve in Fig. 2 is used for the calculation, we find that the relative activity at pH = 5 is RA = 0.37.

The ionization constant of pyridine was determined in the conventional way and found to be $K_B = 6.3 \times 10^{-6}$; the same value is given by Albert [7]. This value for K_B, the value 1.6×10^{-7} for K_A and the RA value 0.37 for $[H^+] = 10^{-5}$ were inserted in formula (15) and gave $K_3 = 7.3 \times 10^{-4}$. Using this value the RA values recorded in Table 2 were calculated.

A calculation according to equation (8) gives $K_1 = 2.85 \times 10^{-2}$ for RA = 0.37 at pH = 5. RA values calculated with this value for K_1 and equation (8) are recorded in Table 2. This table and Fig. 2 show that the values calculated according to equation (15) agree tolerably well with the experimental values, whereas the values calculated according to equation (8) show a considerable deviation. Thus the experiments appear to support the assumption that the cation of the inhibitor reacts with the negatively

Table 2. RA for $[Py_{tot}] = 0.124\ M$ at various pH values.

pH	pH regulated by	Incubation time, min	Relative activity		
			Exptl.	Calculated Eqn. (15)	Calculated Eqn. (8)
3.10	HCl	—	0.66	0.74	0.74
3.10	HCl	30	0.67		
3.60	HCl	—	0.70		
4.04	HCl	—	0.67	0.77	0.77
4.10	Acetate	—	0.47		
4.25	HCl	—	0.55		
4.70	HCl	—	0.46	0.49	0.49
4.95	Acetate	—	0.33		
4.95	Acetate	30	0.33		
5.00	—	—	(0.37)	(0.37)	(0.37)
5.15	HCl	—	0.38		
5.30	—	—	(0.32)	0.29	0.29
5.85	HCl	—	0.23		
5.90	Acetate	—	0.25	0.24	0.19
6.30	Phosphate	—	0.26		
6.30	Phosphate	30	0.26		
6.40	Phosphate	—	0.26	0.19	0.14
6.85	Acetate	—	0.19		
6.90	HCl	—	0.19	0.16	0.08
7.55	Acetate	—	0.07	0.09	0.03

charged enzyme. As pointed out in Part XI [5], the choice of the K_A value, 1.6×10^{-7}, may appear arbitrary. However, this value is derived from the alkaline branch of the activity pH curve of yeast invertase. As found originally by Michaelis and Davidsohn [8], this part of the activity pH curve has the form of a simple ionization curve with $K_A \sim 2 \times 10^{-7}$. The value 1.6×10^{-7} was determined with the enzyme used here and under the same experimental conditions as in the inhibition experiments. In the case of the inhibition by aniline [5], other values for K_A were tried but no better agreement with the experimental inhibition pH curve could be found.

Experiments with quinoline

Inhibition experiments with $8.46 \times 10^{-3}\ M$ quinoline were carried out at various pH's (Table 3). The continuous curve in Fig. 3 is the experimental activity pH curve. Inserting the RA value 0.58 for $[H^+] = 10^{-5}$, $K_A = 1.6 \times 10^{-7}$ and $K_B = 1.3 \times 10^{-5}$ [7] in equation (15) we find $K_3 = 8.4 \times 10^{-5}$. Using this value the RA values recorded in Table 3 have been calculated. Fig. 3 shows that experimental and calculated values agree well, i.e. the dependence of the inhibition by quinoline on pH is well explained on the assumption that the quinolinium ion reacts with the "acidic group" of invertase with $K_A = 1.6 \times 10^{-7}$. As in the case of the inhibition by aniline [5], assumption of a reaction of the quinolinium ion with enzyme groups of other K_A values does not lead to agreement with the experimental data.

The assumption of a reaction of the free quinoline base with a non-ionic group of the enzyme gives, according to equation (8), $K_1 = 6.95 \times 10^{-3}$ and the RA values

Table 3. Inhibition by 8.46×10^{-3} *M* quinoline at various pH values. No incubation.

pH	Buffer	Relative activity		
		Exptl.	Calculated Eqn. (15)	Eqn. (8)
4.50	Acetate	0.65	0.73	0.73
5.00	Acetate	0.58	(0.58)	(0.58)
5.50	Phosphate	0.48	0.49	0.49
5.73	Phosphate	0.48	0.46	0.45
6.10	Phosphate	0.46	0.42	0.39
6.25	Acetate	0.40	0.41	0.37
6.70	—	(0.33)	0.33	0.25
7.10	Phosphate	0.25	0.24	0.15
8.00	Phosphate	0.05	0.05	0.03

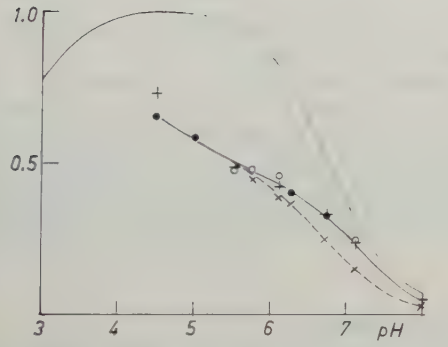

Fig. 3.

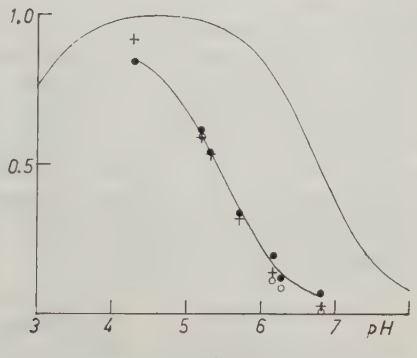

Fig. 4.

Fig. 3. Inhibition pH curves for 8.46×10^{-3} *M* quinoline. ●, acetate buffer; ○, phosphate buffer; +, values calculated from equation (15); ⁄, values calculated from equation (8). Ordinate, relative activity.

Fig. 4. Inhibition pH curves for 0.230 *M* morpholine. ●, experimental values; ○, values calculated from equation (15); +, values calculated from equation (8). Ordinate, relative activity.

recorded in Table 3. Fig. 3 shows that the calculated values deviate considerably from the experimental curve.

Experiments with morpholine

Investigation of the inhibition of yeast invertase by morpholine seemed of interest with regard to the fact that morpholine is a considerably stronger base ($K_B = 2.0 \times 10^{-9}$ [7]) than pyridine and quinoline; the inhibiting action on invertase is relatively weak.

An aqueous morpholine solution was neutralized with HCl to pH about 5; the pH of the assay mixtures was then regulated with acetate buffer. RA was determined for a number of pH's and the morpholine concentration $[Mo_{tot}] = 0.230$ *M* (Table 4). The form of the inhibition pH curve differs strongly (Fig. 4) from those of aniline,

Table 4. Inhibition by 0.230 *M* morpholine in acetate buffer at various pH values. No incubation.

	Relative activity		
		Calculated	
pH	Exptl.	Eqn. (15)	Eqn. (8)
4.30	0.84	0.92	0.92
5.20	0.61	0.59	0.59
5.30	0.54	(0.54)	(0.54)
5.70	0.34	0.32	
6.17	0.20	0.14	0.11
6.25	0.12	0.12	0.09
6.80	0.07	0.03	0.004

pyridine and quinoline. RA is 0.54 for pH = 5.3. Equation (15) gives $K_3 = 9.0 \times 10^{-3}$. The RA values calculated with this value for K_3 and equation (15) are recorded in Table 4. The agreement with the experimental values is good (Fig. 4).

If RA = 0.54 for pH = 5.3 is used to calculate K_1 according to equation (8), we find $K_1 = 1.1 \times 10^{-4}$; corresponding RA values in Table 4. Since K_B is very small compared to $[H^+]$, the two sets of calculated RA values in the table differ markedly only above pH ~ 6, i.e., the experiments can hardly allow a decision between the two mechanisms of inhibition, represented by equations (8) and (15). However, the mechanism corresponding to equation (8) appears to be highly improbable in the case of the inhibition by morpholine.

Experiments with piperidine

Piperidine is a very strong base ($K_B = 6.3 \times 10^{-12}$ [7]); its inhibiting action on yeast invertase was compared to that of the weaker heterocyclic bases. An aqueous solution of piperidine was neutralized to pH ~ 5 with HCl; acetate or phosphate buffers were added to the desired pH values. Final concentration of piperidine in the assay mixtures 0.102 *M* (Table 5). The inhibition by piperidine is considerably stronger than that of morpholine, but the dependence of the inhibition on pH is much the same in both cases.

Since K_B for piperidine is 6.3×10^{-12} it is negligible compared to $[H^+]$ in the pH range investigated, and equation (15) can be used in the simplified form

$$RA = \frac{1}{1 + \frac{K_A}{[H^+]}\left(1 + \frac{0.102}{K_3}\right)}$$

Inserting the value RA = 0.63 for pH = 5.05 we find $K_3 = 3.1 \times 10^{-3}$, and using this value RA was calculated for other pH values (Table 5). As seen from Fig. 5, the agreement between calculated and experimental values is comparatively good. Nevertheless, a certain deviation is present, and an explanation should be found. The type of buffer does not seem to have any marked influence on RA. The value of K_B is so small that even a considerable error in this value cannot materially

Table 5. Inhibition by 0.102 *M* piperidine at various pH values. No incubation.

pH	Buffer	Relative activity		
		Exptl.	Calculated	
			Eqn. (15)	Eqn. (14)
4.30	Acetate	0.87	0.96	0.88
5.05	Acetate	0.63	(0.63)	(0.63)
6.00	Phosphate	0.29	0.17	0.27
6.08	Acetate	0.25		
6.15	Acetate	0.27	0.12	
6.80	Phosphate	0.12	0.03	0.12

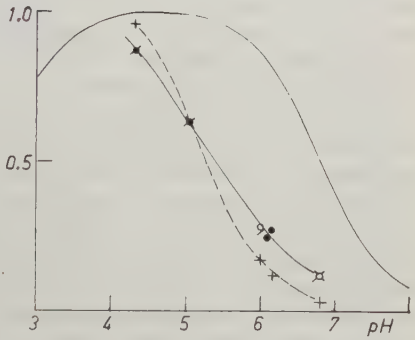

Fig. 5. Inhibition pH curve for 0.102 *M* piperidine. ●, experiments in acetate buffer; ○, experiments in phosphate buffer; +, values calculated from equation (15); ×, values calculated from equation (14). Ordinate, relative activity.

alter the calculated activity values. As pointed out earlier, the value of $K_A = 1.6 \times 10^{-7}$ may appear somewhat arbitrary. In Part XI of this series [5] other values were tested to explain the inhibition by aniline. If it be assumed that the positive ion of the inhibiting base reacts with a group of the enzyme molecule with $K_{A2} = 2 \times 10^{-6}$, RA can be calculated according to equation (14). For RA = 0.63 and pH 5.05 we find $K_6 = 3.0 \times 10^{-2}$ and using this value the RA values, recorded in Table 5 have been calculated. As seen from Fig. 5, these values agree well with the experimental inhibition pH curve. However, the value $K_{A2} = 2 \times 10^{-6}$ is quite arbitrary and the agreement is probably incidental.

If the value RA = 0.63 for pH = 5.05 is used for calculation of K_1 according to equation (8), we get $K_1 = 1.2 \times 10^{-7}$.

Discussion

The strong inhibition of yeast invertase by primary aromatic amines has been regarded as due to a reaction of the free amine base with an aldehyde group of the enzyme to form a Schiff base. The dependence of the inhibition on pH appeared to be in fairly good agreement with this hypothesis. A reinvestigation of the inhibition by aniline and its dependence on pH [5] revealed that a still better agreement between experimental and calculated values is obtained if it be assumed that the anilinium ion reacts with the negatively charged enzyme ($K_A = 1.6 \times 10^{-7}$). As shown in the

Table 6. Dissociation constants of enzyme-inhibitor complexes.

Base	pK_B	pK_3	pK_1
Piperidine	11.2	2.5	6.9
Morpholine	8.7	2.1	4.0
Pyridine	5.3	3.1	1.5
Quinoline	4.9	4.1	2.2
Aniline	4.6	5.5	3.4

present paper, several heterocyclic bases inhibit yeast invertase and the inhibition varies with pH in much the same way as that of aniline. Since the heterocyclic bases cannot be supposed to give Schiff bases with the enzyme, the inhibition by four heterocyclic bases of varying basicity was investigated in detail.

Assuming, (i) that the cations of the bases react with the negatively charged enzyme, dissociation constants, K_3, of the base–enzyme compounds have been calculated (Table 6). Using these constants, the inhibition pH curves were calculated and found to coincide with the experimental curves tolerably well; the agreement was good especially with the weaker bases.

The striking increase of the inhibition of yeast invertase by the organic bases with increasing pH shows that the true inhibitor is *either* the base cation *or* the free base. Although an interaction of the uncharged base with the enzyme appears unlikely, calculations have also been carried out under the assumption (ii) that uncharged base reacts with an uncharged group of the enzyme (e.g., an aldehyde group). The corresponding dissociation constants, K_1, of the enzyme-inhibitor compounds are found in Table 6.

For the strong bases (piperidine, morpholine) calculations of the inhibition pH curves on the basis of the two different assumptions ((i), (ii)) yield, for obvious reasons, practically the same result, whereas for the weak bases (quinoline, pyridine) the results differ markedly in the pH range 5–8. As is seen from Figs. 2 and 3, the inhibition pH curves calculated under assumption (i) agree considerably better with the experiments than those calculated under assumption (ii). The conclusion that the interaction of the enzyme with the heterocyclic bases is essentially ionic, i.e. the formation of salt-like compounds between inhibitor cation and the negatively charged enzyme, appears plausible also from other reasons. It may be mentioned, for instance, that the assumption of an interaction of the uncharged piperidine base with the enzyme is unlikely since the concentration of uncharged piperidine in the pH range in question is extremely low and, in consequence, K_1 would have a value of $10^{-6.9}$, which appears highly unlikely.

SUMMARY

The inhibition of yeast invertase by some heterocyclic bases has been studied. The dependence of the inhibition on pH is best explained by the assumption that the base cations form complexes with the negatively charged enzyme. Inhibition pH curves, calculated on this assumption, agree well with the experiments, especially in the case of the weaker bases. Since the heterocyclic bases cannot be supposed to form Schiff bases with aldehyde groups, the experiments lend sup-

port to the view that also the primary aromatic amines inhibit the enzyme through the formation of salt-like compounds.

The inhibiting power of the heterocyclic bases investigated is considerably weaker than that of aniline. The strong heterocyclic bases are much less inhibitory than the weak ones.

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Proton magnetic resonance of thiophenes

IX. Preparation of substituted thiophenethiols

By Salo Gronowitz, Pinchas Moses[1] and Anna-Britta Hörnfeldt

With 10 figures in the text

Comparatively little is known about the thiophenethiols [1]. Although 2-thiophenethiol was isolated by Victor Meyer [2] as long ago as 1887, as a by-product in the classical thiophene synthesis from succinic acid and phosphorous pentasulphide, active interest in these compounds arose only recently with the discovery that 3-thiophenethiol was a by-product in the commercial synthesis of thiophene from butane and sulphur [3]. 3-Thiophenethiol has also been prepared from 3-thiophenemagnesium bromide and sulphur [3], and more recently by a more convenient method, employing the corresponding lithium intermediate [4].

Brooks *et al.* have prepared the sulphides, mercaptals and esters of 3-thiophenethiol [5]. Houff & Schuetz have prepared the ω-(*N*,*N*-dialkylamino)-alkyl derivatives of 2- [6] and 3-thiophenethiol [7] for a study of their properties as local anaesthetics.

Ring substituted thiophenethiols have until recently been almost completely unknown. 2-Ethyl-3-thiophenethiol has been obtained in the reaction of thieno[3,2-b] thiophene with sodium in alcohol [8]. 5-Methyl-2-thiophenethiol has been detected as a by-product in the reaction of levulinic acid with phosphorous pentasulphide and has been prepared through the reaction of 5-methyl-2-thiophenemagnesium bromide with sulphur [9]. Quite recently, 2,5-dimethyl- and 2,5-dichloro-3-thiophenethiol and 5-phenyl-2-thiophenethiol have been prepared for a study of the pharmacological properties of their ω-(*N*,*N*-dialkylamino)-alkylsulphides [10].

The present authors became interested in the thiophenethiols in connection with a general and extensive study of the NMR-spectra of the thiophenes [11]. 2- and 3-thiophenethiol have been studied previously [12] but the information gained from that study was too scanty to permit a full understanding of the coupling behaviour of the thiol hydrogen. The spectrum of 3-thiophenethiol showed a very complex fine structure, which was impossible to interpret in detail. The spectrum of 2-thiophenethiol, on the other hand, could be completely resolved, but since the nature and mechanism of the thiol couplings could not be completely elucidated from it, a study of the NMR-spectra of a number of substituted thiophenethiols has been undertaken.

Our choice of compounds prepared in the present study was governed by the need to have at our disposal a number of isomeric monosubstituted thiophenethiols in which the various possible coupling positions are blocked with non-interacting

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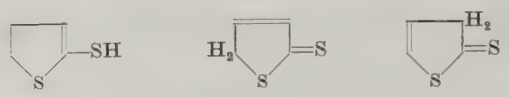
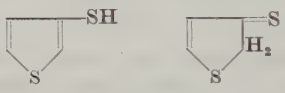
substituents (Br, SCH_3). By studying the NMR-spectra of such compounds, the relative positions of the coupling nuclei can be determined unambiguously [13], and information can be obtained as to the mechanism of spin-spin couplings in the thiophenethiols. Methyl substituted thiophenethiols have also been prepared, as there exists in these compounds the possibility of coupling between the two substituents, the occurrence of such couplings giving further information about the coupling mechanism [9].

A study of the structure of the substituted thiophenethiols is of interest in connection with the possible tautomeric nature of these compounds. Theoretically, 2-thiophenethiol can exist, not only in its thiol form (I), but also in two isomeric thione forms (II and III)



I II III

whilst 3-thiophenethiol can exist in one thiol (IV) and one thione (V) form.



IV V

In his monograph, Hartough [14] cites as unpublished results by Caesar that 3-thiophenethiol exists both in the thiol and thione forms; in neutral and basic solutions[1] the thiol form predominates, but in acid solutions appreciable amounts of the thione form is present. In their paper, however, Caesar & Branton [3] did not bring up this question at all but merely stated that a stretching band due to the thiol group, in addition to bands characteristic of the thiophene nucleus, was apparent in the IR-spectrum of 3-thiophenethiol. This, however, does not exclude the possibility that the thione form is present as well. More decisive information as to the correct structure of 2- and 3-thiophenethiol has been obtained from their NMR-spectra [12], where no bands ascribable to the thione forms could be detected in the pure liquids or in *cyclo*hexane solutions. On the other hand, from the absence of the SH-stretching band in the IR-spectrum of 3,4-thiophenedithiol, Caesar & Branton [3] concluded that this compound exists exclusively in the dithione form.

Thiophenethiols can be conveniently prepared either by reduction of thiophene sulphonyl chlorides [6, 10] or by the reaction of metal substituted thiophenes with sulphur [3, 4]. In the present study, the latter method only has been used, which can be carried out under very mild conditions. With the exception of 3-methyl-2-thiophenethiol, which was prepared from the appropriate Grignard reagent, the thiols were prepared through the reaction of sulphur with the appropriate organolithium compound at $-70°$. The organolithium intermediates were prepared from the corresponding bromo compound, through halogen-metal interconversion with *n*-butyllithium at $-70°$ [15]. The temperature has to be kept low throughout the entire

[1] In basic media, a mesomeric anion common to both the thiol and thione forms should be present.

Table 1. Yields and physical constants of substituted thiophenethiols.

Compound	Yield, %	B.p.	n_D^{20}
3-Methyl-2-thiophenethiol	52	85–86°/30 mm Hg	1.5956
2-Methyl-3-thiophenethiol	62	71–74°/11 mm Hg	1.5978
4-Methyl-3-thiophenethiol	50	76–78°/14 mm Hg	1.6020
5-Methylthio-2-thiophenethiol	64	81–84°/0.4 mm Hg	1.6583
2-Methylthio-3-thiophenethiol	43	71–72°/0.4 mm Hg	1.6500
4-Methylthio-3-thiophenethiol	50	90–91°/0.3 mm Hg	1.6662
5-Methylthio-3-thiophenethiol	55	93–96°/0.7 mm Hg	1.6593
3-Bromo-2-thiophenethiol	59	82–85°/1.0 mm Hg	1.6579
4-Bromo-2-thiophenethiol	51	74–75°/0.4 mm Hg	1.6560
4-Bromo-3-thiophenethiol	67	91–93°/1.5 mm Hg	1.6642

synthesis in order to avoid alkylation of the lithium salt of the thiol formed by the alkyl halide formed in the interconversion (cf. [16]). The yields obtained were 43–67 %. The isomeric purity of the thiols was established through IR- and NMR-spectroscopy. Some physical data of the thiols prepared are listed in Table 1.

Marked differences in the stability of the different thiols are noted. Whilst the methylthio- and especially the methylthiophenethiols appear to be rather stable,[1] the bromothiophenethiols were very unstable, some of them decomposing with evolution of hydrogen bromide and hydrogen sulphide within 30 minutes of their isolation by vacuum distillation. Several attempts to prepare 5-bromo-2-thiophenethiol failed because the compound decomposed during distillation. It seems that an α-bromine increases the instability of these compounds. However, our evidence is not conclusive, since the preparation of only one α-bromothiophenethiol has been attempted in the present study.

The instability of the bromothiophenethiols is unexpected and in strong contrast to the apparent stability of the bromothiophenols [17–19]. In view of this remarkable instability, it is not surprising that all attempts by Profft & Kubat [20] to prepare 2,4-dichloro-5-thiophenethiol by reducing 2,4-dichloro-5-thiophenesulphonyl chloride with a great variety of reducing agents failed.

The relative instability in general of the thiophenethiols leads us to the tempting hypothesis that the possibility of these compounds to revert to the thione form might be a contributory factor to the high instability of the bromothiophenethiols. The greater instability of these compounds in comparison to the methyl- and methylthiothiophenethiols might be explained on the basis of the bromo compounds possessing an aliphatic halogen in some of the thione forms. That no thione form has been detected in the NMR-spectra of these compounds does not invalidate this hypothesis since the equilibrium mixture might contain only undetectable amounts of the thione form.

It has been observed that other thiophenes capable of existing in more than one tautomeric form are also unstable. In the aminothiophenes [21] it has been noted that the 3-isomer is more unstable than the 2-isomer, an observation which is paralleled in the hydroxythiophenes [22]. We have found similar differences in the stability of 2- and 3-thiophenethiol.

[1] A sample of 5-methyl-2-thiophenethiol [9] gave unchanged IR- and NMR-spectra after an interval of eight months.

The IR-spectra of the ten analytically pure thiols prepared all show the SH-stretching band at 3.94–3.99 μ (2538–2506 cm^{-1}). They also show ring-stretching frequencies in the region characteristic of the thiophene nucleus [23]. In the region between 10–15 μ, they show the pattern characteristic for the different types of disubstituted thiophenes. Thus all the 3,4-disubstituted thiophenes show peaks at 10.80–11.00 μ (926–909 cm^{-1}), at 11.65–11.95 μ (858–836 cm^{-1}) and at 12.80–12.90 μ (781–775 cm^{-1}). Similarly, the strong band at 14.0–14.04 μ (714–694 cm^{-1}) observed in many 2,3-disubstituted thiophenes is present in the 2,3-substituted thiols as well, and the peaks at 12.10–12.40 μ (826–806 cm^{-1}) characteristic of 2,4-disubstituted thiophenes appear in the 2,4-substituted thiophenethiols also.[1]

From the NMR-spectra of the thiols, it is evident that they exist in the thiol form in the pure liquid state as well as in *cyclo*hexane solution. A detailed discussion of the NMR-spectra of the thiols and of their coupling behaviour is given elsewhere [25].

Experimental

3-Methyl-2-thiophenethiol. To the Grignard reagent prepared in the usual apparatus from 17.7 g (0.10 moles) of 3-methyl-2-bromothiophene [26] in 100 ml of absolute ether and 2.6 g (0.11 moles) of magnesium was added, after cooling to 0°, 3.2 g (0.10 moles) of dry sulphur in one portion. After refluxing for 1 hour, the reaction mixture was cooled and decomposed with 90 ml of 6-*N* hydrochloric acid. The aqueous phase was extracted once with ether and the combined ether phases were extracted three times with 50 ml portions of 2-*N* potassium hydroxide solution. The alkaline phase was acidified under cooling with 2-*N* sulphuric acid and the thiol immediately extracted with ether. The combined ether phases were washed with water, dried over sodium sulphate and fractionated at reduced pressure to give 6.8 g (52 %) of 3-methyl-2-thiophenethiol, b.p. 85–86°/30 mm Hg, $n_D^{20} = 1.5956$, IR-spectrum (Fig. 1).

Anal. $C_5H_6S_2$ (130.2)			
Calc.	C 46.12	H 4.64	S 49.24
Found	C 46.08	H 4.71	S 48.94

4-Methyl-3-thiophenethiol. A solution of 16 g (0.09 moles) of 4-bromo-3-methylthiophene [15] in 30 ml of dry ether was added to a stirred solution of 84 ml of 1.20-*N* *n*-butyllithium at −70° in the usual four-necked nitrogen-swept flask. After stirring at −70° for 5 minutes, 3.2 g (0.10 moles) of dry sulphur was added in one portion, and stirring continued at −70° for half an hour. The product was hydrolyzed with water, the aqueous layer separated and the ether layer immediately extracted three times with 25 ml portions of 2-*N* sodium hydroxide solution. The combined aqueous phases were acidified under cooling with 2-*N* sulphuric acid and immediately extracted with ether. The ether layer was washed with water, dried over sodium sulphate, the ether distilled off and the residue fractionated *in vacuo* over nitrogen. 5.7 g (50 %) of crude thiol, b.p. 75–84°/14 mm Hg (mainly 80°) was obtained. Redistillation gave pure 4-methyl-3-thiophenethiol, b.p. 76–78°/14 mm Hg, $n_D^{20} = 1.6020$. In its IR-spectrum (Fig. 2) the peaks of 3-methyl-2-thiophenethiol at 8.17 μ (1224 cm^{-1}) and 12.04 μ (830 cm^{-1}) were absent.

[1] Though it is possible to take advantage of these characteristic IR-patterns, the best way of characterizing disubstituted thiophenes is by means of their ring-coupling constants in the NMR-spectra [16, 24, 25].

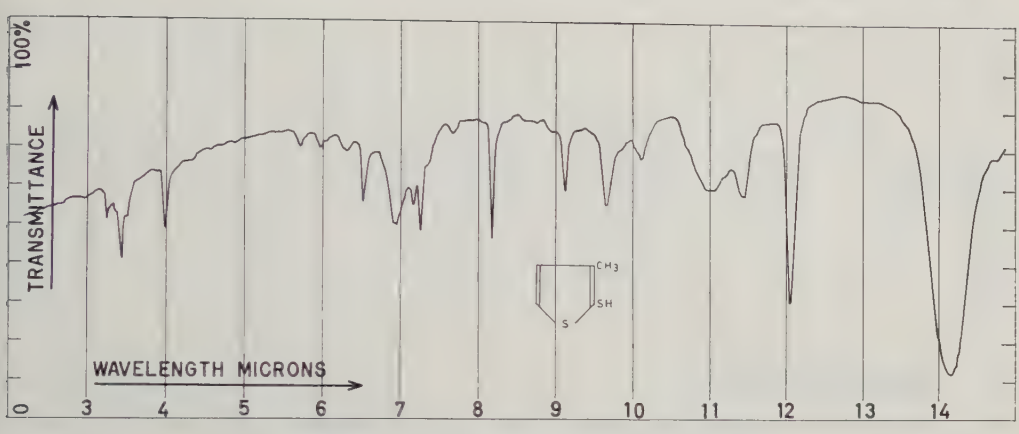


Fig. 1. IR-spectrum of 3-methyl-2-thiophenethiol.

Anal. $C_5H_6S_2$ (130.2)

Calc.	C 46.12	H 4.64	S 49.24
Found	C 45.98	H 4.78	S 49.10

2-Methyl-3-thiophenethiol. This was prepared from 14.8 g (0.083 moles) of 2-methyl-3-bromothiophene [15] in 75 ml of ether, 83 ml of 1.11-*N* *n*-butyllithium and 2.7 g (0.084 moles) of sulphur. The procedure followed was as described above, except that the *n*-butyllithium was added to the methyl-bromothiophene. Fractionation yielded 7.3 g (67 %) of the crude thiol, b.p. 74–82°/16 mm Hg. Redistillation gave 6.8 g (62 %) of pure 2-methyl-3-thiophenethiol, b.p. 71–74°/11 mm Hg, $n_D^{20} = 1.5978$. In its IR-spectrum (Fig. 3) the peaks of 5-methyl-2-thiophenethiol at 8.24 μ (1213 cm^{-1}) and at 9.40 μ (1064 cm^{-1}) [9] were absent.

Anal. $C_5H_6S_2$ (130.2)

Calc.	C 46.12	H 4.64	S 49.24
Found	C 46.12	H 4.72	S 49.16

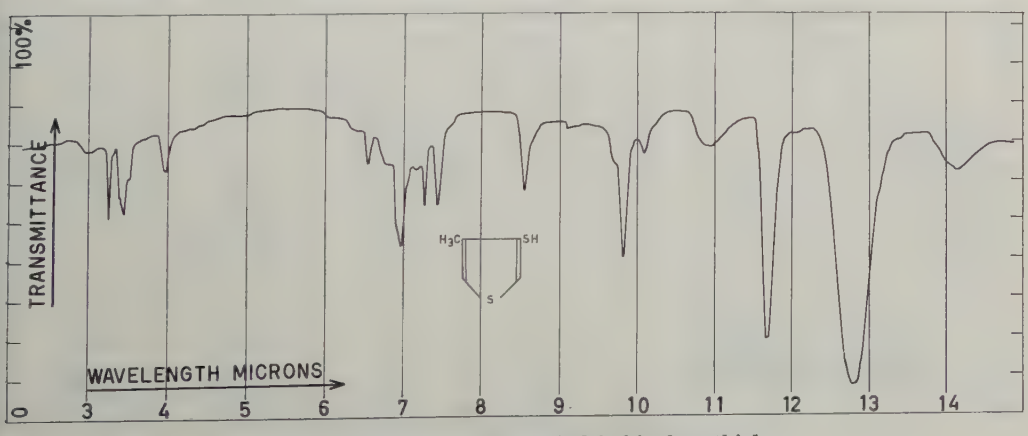


Fig. 2. IR-spectrum of 4-methyl-3-thiophenethiol.

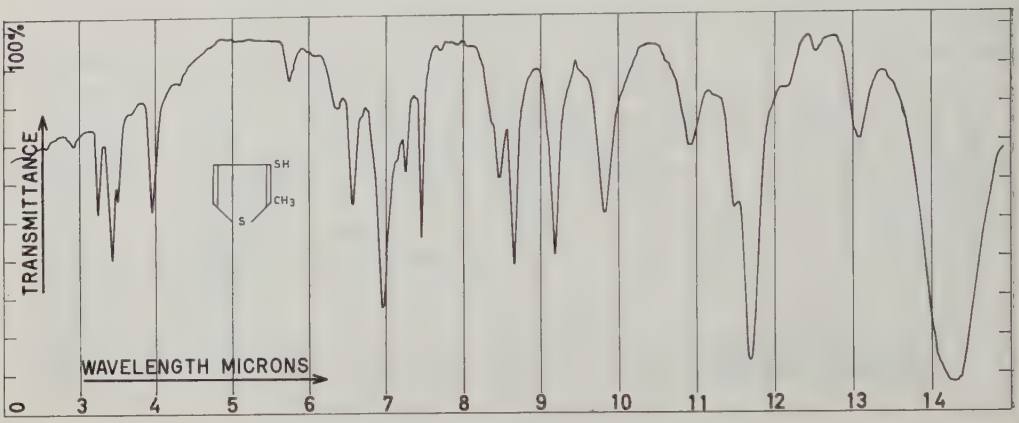


Fig. 3. IR-spectrum of 2-methyl-3-thiophenethiol.

4-Methylthio-3-thiophenethiol. This was prepared in an analogous manner to 4-methyl-3-thiophenethiol, from 14.8 g (0.071 moles) of 4-bromo-3-methylthio-thiophene [15], 60 ml of 1.30-*N* *n*-butyllithium and 2.4 g (0.075 moles) of sulphur. Fractionation yielded 7.2 g (63 %) of the crude thiol, b.p. 94–102°/0.4 mm Hg. Redistillation gave 5.8 g (50 %) of pure 4-methylthio-3-thiophenethiol, b.p. 90–91°/0.3 mm Hg, $n_D^{20} = 1.6662$, IR-spectrum (Fig. 4).

Anal. $C_5H_6S_3$ (162.3)			
Calc.	C 37.01	H 3.72	S 59.27
Found	C 37.21	H 3.81	S 58.58

5-Methylthio-3-thiophenethiol. This was prepared as described above from 20.0 g (0.096 moles) of 4-bromo-2-methylthio-thiophene [15] in 50 ml of ether, 80 ml of 1.35-*N* *n*-butyllithium and 3.3 g (0.10 moles) of sulphur. Fractionation yielded 8.6 g (55 %) of pure 5-methylthio-3-thiophenethiol, b.p. 93–96°/0.7 mm Hg, $n_D^{20} =$

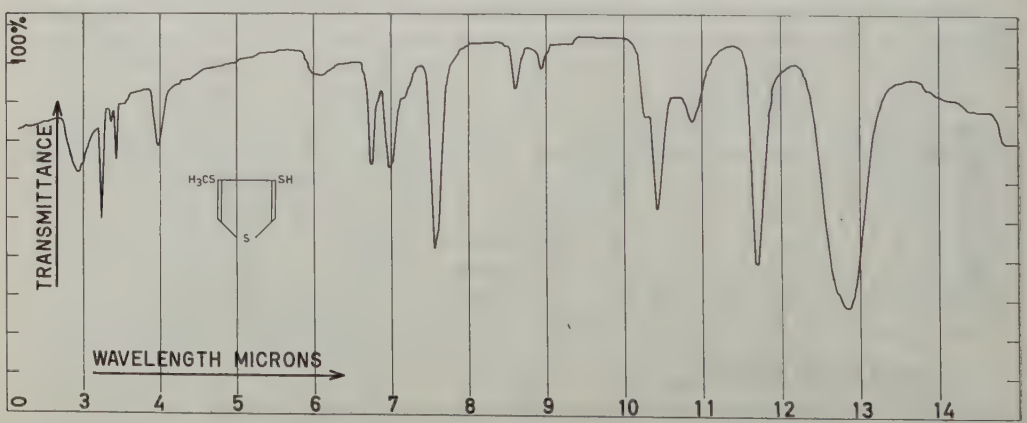


Fig. 4. IR-spectrum of 4-methylthio-3-thiophenethiol.

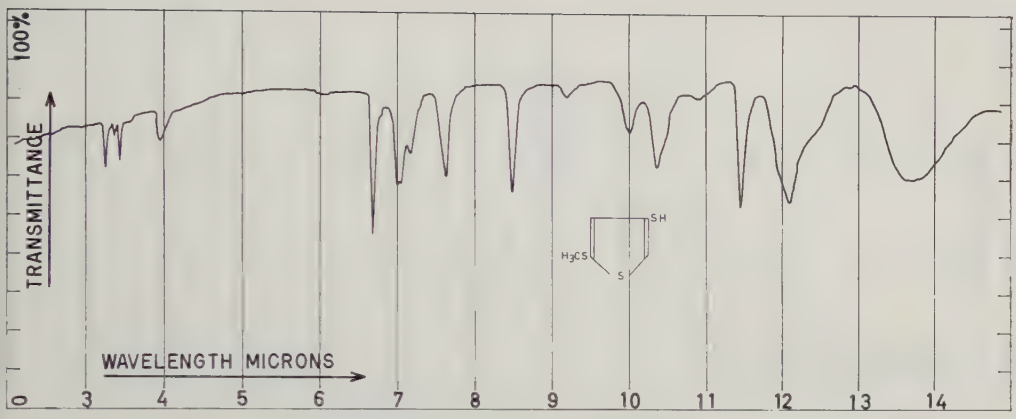


Fig. 5. IR-spectrum of 5-methylthio-3-thiophenethiol.

1.6593. In its IR-spectrum (Fig. 5) the peaks of 2-methylthio-3-thiophenethiol at 14.05 μ (712 cm^{-1}) and of 5-methylthio-2-thiophenethiol at 8.32 μ (1202 cm^{-1}) and at 12.55 μ (797 cm^{-1}) were absent.

Anal. $C_5H_6S_3$ (162.3)

Calc.	C 37.01	H 3.72	S 59.27
Found	C 37.05	H 3.80	S 58.93

2-Methylthio-3-thiophenethiol. This was prepared as described above from 14.1 g (0.067 moles) of 2-methylthio-3-bromothiophene [15] in 50 ml of ether, 57 ml of 1.30-*N* *n*-butyllithium and 2.2 g (0.069 moles) of sulphur, the butyllithium being added to the methylthio-bromothiophene. Distillation yielded 4.7 g (43 %) of pure 2-methylthio-3-thiophenethiol, b.p. 71–72°/0.4 mm Hg, $n_D^{20} = 1.6500$. In its IR-spectrum (Fig. 6) the peaks of 5-methylthio-2-thiophenethiol at 8.32 μ (1202 cm^{-1}) and at 12.55 μ (797 cm^{-1}) and of 5-methylthio-3-thiophenethiol at 10.02 μ (998 cm^{-1}) were absent.

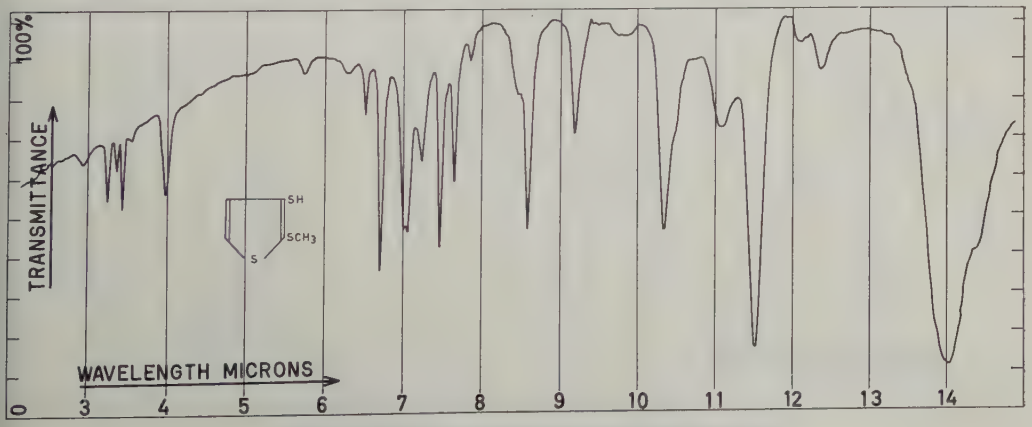


Fig. 6. IR-spectrum of 2-methylthio-3-thiophenethiol.

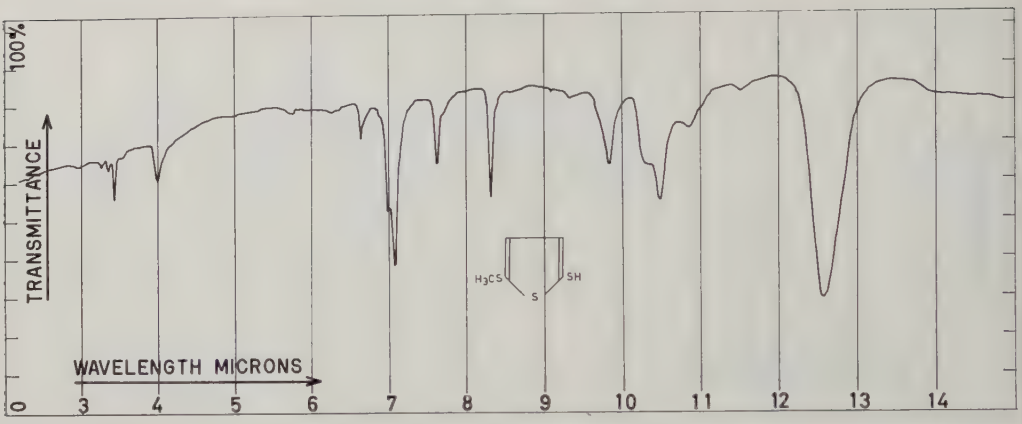


Fig. 7. IR-spectrum of 5-methylthio-2-thiophenethiol.

Anal. $C_5H_6S_3$ (162.3)

Calc.	C 37.01	H 3.72	S 59.27
Found	C 37.11	H 3.78	S 58.98

5-Methylthio-2-thiophenethiol. This was prepared as described above from 26.8 g (0.13 moles) of 5-bromo-2-methylthio-thiophene [15] in 75 ml of ether, 110 ml of 1.35-*N* *n*-butyllithium and 4.6 g (0.14 moles) of sulphur. Distillation yielded 14.6 g (69 %) of crude thiol, b.p. 78–84°/0.4 mm Hg. Refractionation gave 13.5 g (64 %) of pure 5-methylthio-2-thiophenethiol, b.p. 81–84°/0.4 mm Hg, $n_D^{20} = 1.6583$. In its IR-spectrum (Fig. 7) the peaks of 5-methylthio-3-thiophenethiol at 8.48 μ (1179 cm^{-1}) and at 11.45 μ (873 cm^{-1}) and of 2-methylthio-3-thiophenethiol at 8.58 μ (1166 cm^{-1}) and at 14.05 μ (712 cm^{-1}) were absent.

Anal. $C_5H_6S_3$ (162.3)

Calc.	C 37.01	H 3.72	S 59.27
Found	C 37.10	H 3.84	S 58.90

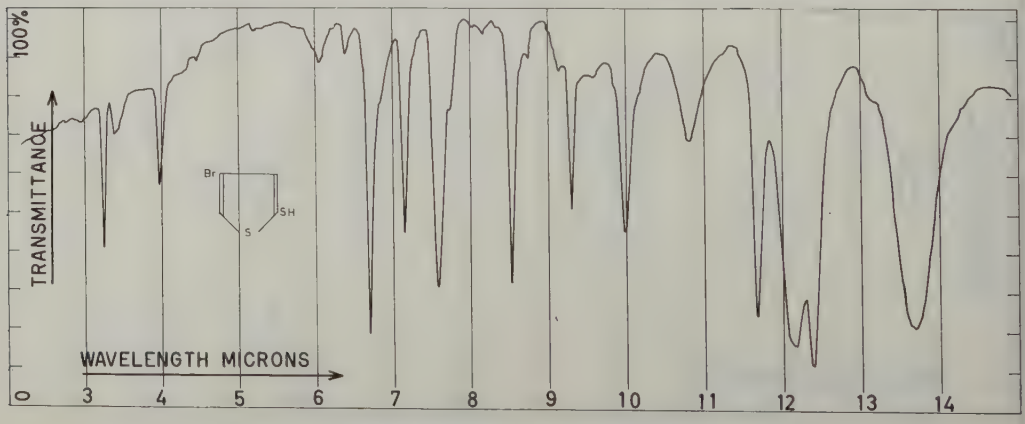


Fig. 8. IR-spectrum of 4-bromo-2-thiophenethiol.

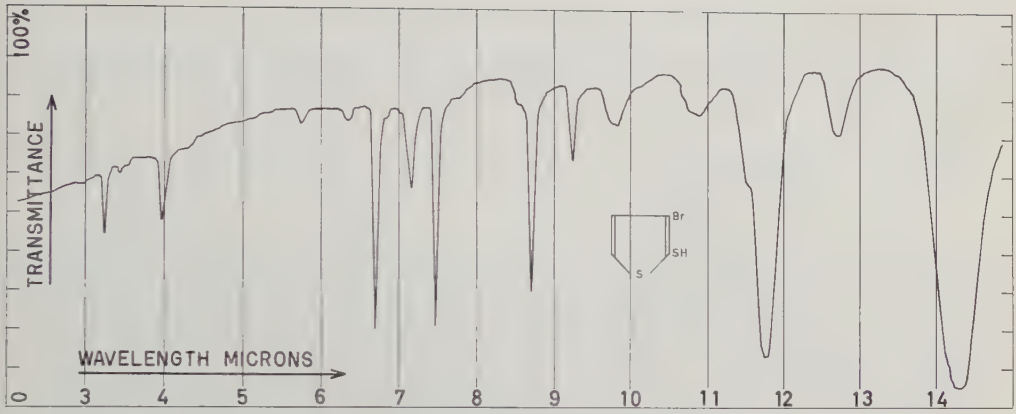


Fig. 9. IR-spectrum of 3-bromo-2-thiophenethiol.

4-Bromo-2-thiophenethiol. This was prepared as described above from 34.5 g (0.14 moles) of 2,4-dibromothiophene [27] in 60 ml of ether, 120 ml of 1.35-*N* *n*-butyllithium and 5.0 g (0.16 moles) of sulphur. Fractionation yielded 14 g (51 %) of pure 4-bromo-2-thiophenethiol, b.p. 74–75°/0.4 mm Hg, $n_D^{20} = 1.6560$. In its IR-spectrum (Fig. 8) the peak of 3-bromo-2-thiophenethiol at 14.30 μ (699 cm^{-1}) was absent. This compound was very unstable, decomposing with vigorous evolution of hydrogen bromide and hydrogen sulphide within a few hours, even when kept at −15°.

Anal. $C_4H_3BrS_2$ (195.1)

Calc.	C 24.62	H 1.55
Found	C 25.07	H 1.75

3-Bromo-2-thiophenethiol. This was prepared as described above from 25.4 g (0.105 moles) of 2,3-dibromothiophene [28] in 50 ml of ether, 80 ml of 1.40-*N* *n*-butyllithium and 3.5 g (0.11 moles) of sulphur. Fractionation yielded 12.0 g (59 %) of pure 3-bromo-2-thiophenethiol, b.p. 82–85°/1.0 mm Hg, $n_D^{20} = 1.6579$. In its IR-spectrum

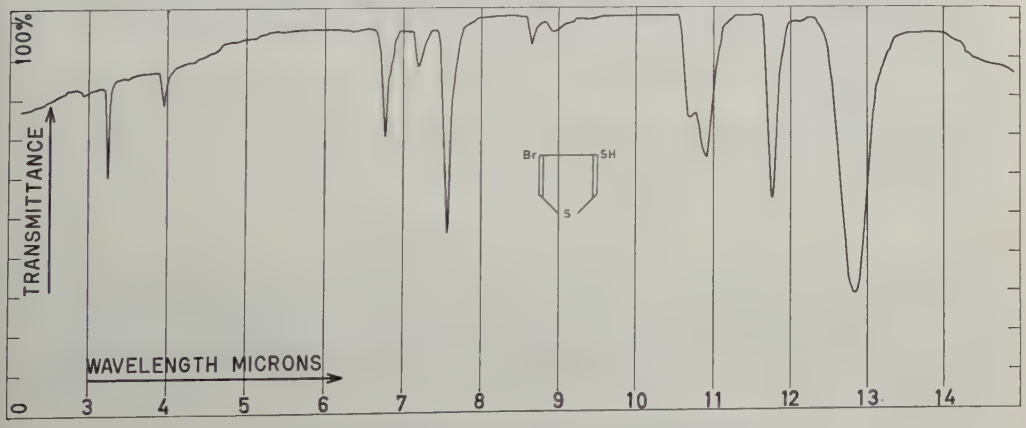


Fig. 10. IR-spectrum of 4-bromo-3-thiophenethiol.

(Fig. 9) the peaks of 4-bromo-2-thiophenethiol at 12.37 μ (808 cm^{-1}) and at 13.67 μ (731.5 cm^{-1}) were absent. This compound was very unstable, decomposing with vigorous evolution of hydrogen bromide and hydrogen sulphide within a few hours, even when kept at $-15°$.

Anal. $C_4H_3BrS_2$ (195.1)

Calc.	C 24.62	H 1.55
Found	C 24.65	H 1.62

4-Bromo-3-thiophenethiol. This was prepared as described above from 24.2 g (0.10 moles) of 3,4-dibromothiophene [15], 92 ml of 1.20-*N* *n*-butyllithium and 3.5 g (0.11 moles) of sulphur, the bromothiophene being added to *n*-butyllithium. Fractionation yielded 13.0 g (67 %) of pure 4-bromo-3-thiophenethiol, b.p. 91–93°/1.5 mm Hg, $n_D^{20} = 1.6642$. In its IR-spectrum (Fig. 10) the peaks of 3-bromo-2-thiophenethiol at 14.30 μ (699 cm^{-1}) and of 4-bromo-2-thiophenethiol at 12.37 μ (808 cm^{-1}) and at 13.67 μ (731.5 cm^{-1}) were absent. The product is unstable, decomposing after some days with evolution of hydrogen bromide and hydrogen sulphide, even when kept at $-15°$.

Anal. $C_4H_3BrS_2$ (195.1)

Calc.	C 24.62	H 1.55
Found	C 24.63	H 1.65

A Perkin Elmer model 21 spectrophotometer equipped with a sodium chloride prism was used for the IR-measurements. The NMR-spectra were obtained at 40 Mc/s with a Varian Associates model V-4300 B high resolution NMR-spectrometer and a flux stabilized 12″ electromagnet from the same company.

The recording of the spectra and the analyses were carried out immediately after the distillations.

SUMMARY

Methyl-, methylthio- and bromothiophenethiols have been prepared, through the reaction of the appropriate lithium compounds with sulphur, for a study of their NMR-spectra.

The following new compounds were prepared: 3-methyl-2-thiophenethiol, 2-methyl-3-thiophenethiol, 4-methyl-3-thiophenethiol, 5-methylthio-2-thiophenethiol, 2-methylthio-3-thiophenethiol, 5-methylthio-3-thiophenethiol, 4-methylthio-3-thiophenethiol, 3-bromo-2-thiophenethiol, 4-bromo-2-thiophenethiol and 4-bromo-3-thiophenethiol.

The NMR-spectra show that these compounds exist in the thiol form in the liquid state and in *cyclo*hexane solution.

The bromothiophenethiols are unstable compounds which decompose spontaneously with the evolution of hydrogen bromide and hydrogen sulphide.

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Chemical Institute, University of Uppsala, Sweden, September 1960.

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Electron spin resonance of Cu(II) and Ag(II) dithiocarbamates

By Rune Pettersson and Tore Vänngård

With 4 figures in the text

Electron spin resonance (ESR) measurements have been shown to be of great value for the study of the bonding of transition metal ions. Cu has probably been most extensively studied, one of the reasons being, that the g-values and hyperfine structure constants give good information about the bonding of the metal ion. Furthermore, the ESR spectra and theory of Cu(II) are not too complicated, as the electron configuration corresponds to a single unpaired "hole".

During the last few years much interest has been devoted to the study of covalent Cu complexes with O and N as coordinating atoms [1–5]. A good example is the investigation by Maki and McGarvey on Cu(II) bis-acetylacetonate [2] and Cu(II) bis-salicylaldehyde-imine [3], where the hyperfine structure of the ligand atoms played an important role. Such studies also form the essential background for the interpretation of ESR spectra of more complicated systems, such as metal proteins [6].

The bonding of Cu(II) with sulphur is likely to be even more covalent than the bonding with O or N, and it was thought to be of interest to determine the ESR parameters of a sulphur compound. The coordination to S might also be of importance in some biological substances [7]. However, only very few stable organic compounds exist with Cu(II) bonded to S; the Cu(I) compounds are usually more stable. Remarkable exceptions are the N, N-dialkyldithiocarbamates of of Cu(II) (Fig. 1), which are quite stable and well-defined [8]. A preliminary report on their ESR spectra has been given in a previous publication [9], and the studies have now been extended to the ligand hyperfine structure and to a determination of the bonding parameters.

The dithiocarbamates also stabilize the divalent state of the other coinage metals, Ag and Au. Their ground state electron configurations are $4d^9$ and $5d^9$ respectively and consequently their magnetic properties should be similar to those of Cu(II) ($3d^9$). Unfortunately, the concentration of the Au(II) form is always small compared to the concentration of Au in other coloured valency states. The optical transitions of Au(II) are not detectable and therefore the properties of the bond cannot be obtained from the ESR data. Only the isotropic hyperfine interaction of Au(II) will be discussed.

The number of stable Ag(II) compounds is much less than the number of Cu(II) compounds, and the available data on ESR of Ag(II) are rather limited [10–12]. Therefore we have chosen to study an Ag(II) dithiocarbamate in some detail, in an attempt to get some information about the bonding.

Theoretical

General

The theory of ESR of the $3d$ transition elements is in part also applicable to $4d$ elements and can briefly be described as follows. A crystal field of appropriate symmetry is applied to the free ion (spin-orbit coupling not included). The field partly (or wholly) removes the orbital degeneracy. For a d^9 configuration a cubic field with a tetragonal distortion usually leaves the unpaired "hole" in the orbital $d_{x^2-y^2}$. A perturbation, consisting of the spin-orbit coupling energy, the magnetic energy and the hyperfine interaction energy, is then applied to the ground state orbital, which is split up into Zeeman sublevels. The experimental data from the observed transitions between these sublevels are conveniently summarized in a so-called spin Hamiltonian. The parameters in the spin Hamiltonian are in our case the g-value defined through the equation

$$h\nu = g\beta_0 B$$

and by hyperfine structure constants. Before the spin-orbit coupling is taken into account, the orbital motion is completely quenched for an orbital singlet, and the g-value is equal to the free electron value 2.0023. The spin-orbit coupling causes the g-value to depart from 2.0023. The g-value and hyperfine structure (hfs) will usually be anisotropic in a way that reflects the symmetry of the crystal field.

ESR of covalent complexes

When the orbital of the magnetic electron is involved in a covalent bond, the unpaired electron is delocalized, so that the probability of finding it at the central ion is less than unity. One effect of this is to reduce the effective spin-orbit coupling, so that the g-value will be closer to 2.0023. (Free radicals, where the unpaired electron is strongly delocalized, have g-values very close to 2.0023.) The coupling with the central nucleus will also be affected by covalent bonding, but in a less predictable way.

Theories for the effect of covalent bonding have been given by Stevens [13] ($4d$ and $5d$ transition groups), Tinkham [14] (iron group fluorides) and by Maki and McGarvey [2, 3] (Cu(II)). The delocalization is treated by introducing molecular orbitals, formed by linear combinations of the central ion orbitals and ligand orbitals. Our formulation will closely follow that of Maki and McGarvey [2, 3].

The four S atoms surrounding the metal ion in the dithiocarbamates do not form a perfect square [15], but for simplicity the crystal field on the central ion is assumed to have full tetragonal symmetry. Simple symmetry considerations show, that the antibonding orbitals of a d^9 configuration can then be chosen as follows, where the unpaired hole is in the $|0\rangle$ orbital:

$$|4\rangle = \beta d_{yz} - \sqrt{1-\beta^2}\,\frac{1}{\sqrt{2}}(p_z^b - p_z^d),$$

$$|3\rangle = \beta d_{xz} - \sqrt{1-\beta^2}\,\frac{1}{\sqrt{2}}(p_z^a - p_z^c),$$

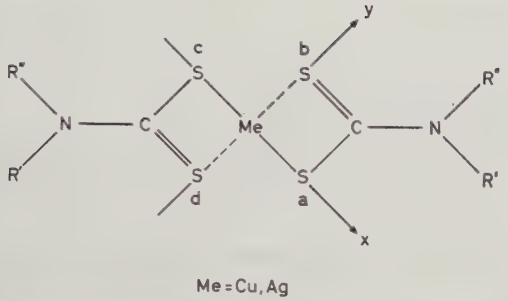


Fig. 1. Formula of the *N*, *N*-dialkyldithiocarbamates. Coordinate system and its relation to the S atoms *a*, *b*, *c* and *d*.

$$|2\rangle = \alpha_1 d_{z^2} - \alpha_1' \tfrac{1}{2}(\sigma_x^a + \sigma_y^b - \sigma_x^c - \sigma_y^d),$$
$$|1\rangle = \beta_1 d_{xy} - \sqrt{1-\beta_1^2}\,\tfrac{1}{2}(p_y^a + p_x^b - p_y^c - p_x^d),$$
$$|0\rangle = \alpha d_{x^2-y^2} - \alpha' \tfrac{1}{2}(-\sigma_x^a + \sigma_y^b + \sigma_x^c - \sigma_y^d).$$

The coordinate system and the denoting of the coordinating atoms is apparent from Fig. 1.

The σ's are normalized ligand hybrids. The actual choice of hybrids should be done under consideration of what is known about the bonding of S in this molecule. The valency angle is probably not much different from 90° [15], which indicates that pure p orbitals should be used. On the other hand, the S^{33} isotropic hyperfine structure to be discussed later suggests, that s electrons are involved in the bonds. Two equivalent hybrids with 90° valency angle can be formed, if a sulphur $3d$ orbital also is included (for example, *spd* hybrids have been proposed for H_2S [16]). The inclusion of $3d$ orbitals would, however, complicate the calculations considerably, and therefore we use the simpler *sp* hybrids:

$$\sigma_x = n\,p_x \pm \sqrt{1-n^2}\,s.$$

The value of n is chosen to give agreement between the calculated and observed S^{33} hfs.

Overlap is only included in the σ bonds, and normalization of $|0\rangle$ gives a relation between α, α' and the overlap integral S (and correspondingly for $|2\rangle$).

If the perturbation applied to the ground state orbital $|0\rangle$ is carried out to second order, a spin Hamiltonian is obtained, which relates the bonding parameters to the experimental ESR data as summarized in the parameters $g_{\|}$, $g_{\perp}$, A and B. $g_{\|}$ and A and $g_{\perp}$ and B are respectively the g-values and hfs constants with the external magnetic field parallel and perpendicular to the symmetry axis. It turns out that the parameters of orbital $|2\rangle$ do not enter the relations, and thus we can only determine α, β_1 and β. A fourth parameter, $\varkappa$, is introduced to allow for the isotropic hyperfine coupling with the central nucleus, which has been explained as arising from mixing with a configuration having unpaired s electrons [17; cf. 18].

The relations are [2]:

$$g_{\|} = 2.0023 - 8\lambda_0 \alpha^2 \beta_1^2/\Delta_1 + 4\vartheta_1,$$

$$g_{\perp} = 2.0023 - 2\lambda_0 \alpha^2 \beta^2/\Delta_2 + \sqrt{2}\,\vartheta_2,$$

$$A = P\left[-\frac{4}{7}\alpha^2 - \varkappa + (g_{\|} - 2.0023) + \frac{3}{7}(g_{\perp} - 2.0023) - 4\vartheta_1 - \frac{3\sqrt{2}}{7}\vartheta_2\right],$$

$$B = P\left[+\frac{2}{7}\alpha^2 - \varkappa + \frac{11}{14}(g_{\perp} - 2.0023) - \frac{11\sqrt{2}}{14}\vartheta_2\right],$$

where

$$\vartheta_1 = \lambda_0 \alpha \alpha' \beta_1 \sqrt{1-\beta_1^2}\, T(n)/\Delta_1,$$

$$\vartheta_2 = \lambda_0 \alpha \alpha' \beta \sqrt{1-\beta^2}\, T(n)/\Delta_2,$$

$$P = 2 g_I \beta_0 \beta_N \langle r^{-3} \rangle_0 \mu_0/4\pi,$$

$$T(n) = n + \tfrac{1}{2}\sqrt{1-n^2}\, R\,\Omega,$$

$$\Omega = \frac{2}{\sqrt{3}} \int_0^{\infty} r^2 R_{31}(r) \frac{d}{dr} R_{30}(r)\, dr.$$

It may be noted that integrals of the type $\langle$ central ion orbital $|\lambda_0 \boldsymbol{L}|$ ligand orbital$\rangle$, where λ_0 is the spin-orbit coupling parameter of the free ion, have been neglected. The spin-orbit coupling probably decreases very rapidly with increasing distance from the central nucleus (cf. [14]). λ_0 is chosen to have the values -829 cm^{-1} and -1843 cm^{-1} for Cu and Ag respectively [19]. Δ_1 is the energy difference between the orbital states $|0\rangle$ and $|1\rangle$, and Δ_2 is the energy difference between $|0\rangle$ and the degenerate states $|3\rangle$ and $|4\rangle$. As the expectation value of $1/r^3$ of the free ion, $\langle r^{-3} \rangle_0$, we have for Cu(II) used the estimate given by Abragam and Pryce [20]. They obtained approximately the same value, 7.25 atomic units, from an integration of a SCF wave function and from the observed hfs in optical spectra. No good wave function is available for Ag, and no optical hfs has been reported for the configurations of Ag, which are of interest here. We can, however, get a rough estimate of $\langle r^{-3} \rangle_0$ from the relation connecting $\langle r^{-3} \rangle_0$ with the fine structure separation $\delta \tilde{W}$ and the effective nuclear charge Z_i [21]:

$$\langle r^{-3} \rangle_0 = \frac{4\pi}{\mu_0} \cdot \frac{\delta \tilde{W}}{2\beta_0^2 (l + \tfrac{1}{2}) Z_i H_r}$$

H_r is a relativistic correction, which in our case is close to unity [21]. Table 1 summarizes the values of the screening number, $Z - Z_i$, for d^9 configurations, where optical hfs and nuclear magnetic moments have been measured by independent means. Barnes and Smith [24] have suggested, that there is a linear relationship between the screening number of p electrons and the principal quantum number, and if this is true for d electrons also, we get $Z - Z_i = 14$ for Ag $3d^9\, 4s^2$. If the influence of the $4s$ electrons on $Z - Z_i$ is neglected and the spectroscopically determined value of $\delta \tilde{W} = 4607$ cm^{-1} [19] is used, we obtain

Table 1. Screening number for d^9 configurations.

Config.	$3d^9\ 4s^2$		$5d^9\ 6s^2$	
Element	Cu		Hg	Tl
Level	$^2D_{5/2}$	$^2D_{3/2}$	$^2D_{5/2}$	$^2D_{3/2}$
$Z-Z_i$	8[a]	11[a]	21[b]	17[b]

[a] Optical hfs: Ritsehl [22]. Nuclear magnetic moments: see, for instance, [21], p. 437.
[b] From v. Siemens [23].

$\langle r^{-3}\rangle_0 = 9.5$ a.u. for Ag(II). The following values of P will be used in the calculations:

$$P_{Cu} = 0.035\ cm^{-1},$$
$$P_{Ag} = -0.0074\ cm^{-1},$$
$$P_{Au} = 0.0041\ cm^{-1}.$$

For Ag we have used an average of the nuclear g-factors ($g_I = -0.243$) (see, for instance, [21], p. 437), and the value for Au is obtained from the optical hfs [23]. The effect of unpaired s electrons on the optical hfs [25] has been neglected in obtaining the P values.

The available X-ray data suggest (cf. [15]) that the internuclear distance R between the Cu(II) ion and the S atoms is 2.4 Å. For the Ag compounds we use $R = 2.55$ Å. The overlap integral S between the central Cu $3d_{x^2-y^2}$ orbital and the ligand orbital $\frac{1}{2}(-\sigma_x^a + \sigma_y^b + \sigma_x^c - \sigma_y^d)$ has been estimated using Slater orbitals, and the result is $S_{Cu} = 0.30$. S_{Ag} is probably not much different, and we have used the same value, $S_{Ag} = 0.30$.

R_{30} and R_{31} in the expression for Ω are S $3s$ and $3p$ radial wave functions respectively. With hydrogen-like wave functions we get

$$\Omega = \frac{128}{3\sqrt{6}} \frac{(Z_s Z_p)^{5/2}(-3Z_s^2 + 14 Z_s Z_p - 3Z_p^2)}{(Z_s + Z_p)^7} (Z_s - Z_p)\ \text{a.u.}$$

Ω, as seen, depends critically on the difference between the effective nuclear charges Z_s and Z_p. We have chosen $Z_s = 7.5$ and $Z_p = 6.0$. A justification for such a choice is that Froese [26] obtains approximately the same difference between the limiting screening numbers of a $3p^4$ configuration.

ESR in solutions

When a paramagnetic molecule is tumbling in a liquid at a sufficient rate, the anisotropic parts of the spin Hamiltonian are averaged out so that only the isotropic parts remain [27]:

$$H = g_s \beta_0 B S_z + a\,\mathbf{S}\cdot\mathbf{I}$$

where $$g_s = (g_{||} + 2g_\perp)/3 \text{ and } a = (A + 2B)/3.$$

It is easily seen that the following relation is obtained:

$$a = P(-\varkappa + g_s - 2) + \text{smaller terms} \qquad (1)$$

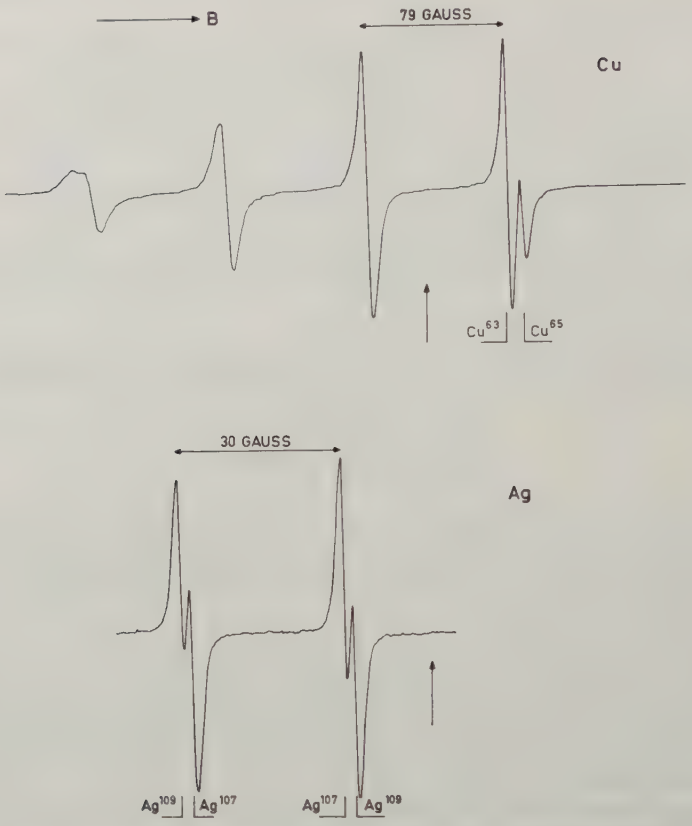


Fig. 2. ESR spectra of deoxygenated benzene solutions of $[(C_2H_5)_2NCS_2]_2Cu$ and of $[(iso\text{-}C_3H_7)_2NCS_2]_2Ag$. The arrows in this and the following figure indicate the resonance field of free electrons.

The transition energies are

$$h\nu = g_s\beta_0 B + a\, m_I + \frac{a^2}{2\, g_s\beta_0 B}[I(I+1) - m_I^2] + \ldots, m_I = -I, \ldots, +I \qquad (2)$$

With insufficient tumbling rate, the width of the resonance lines will be dependent on m_I, as is clearly seen in the Cu spectrum (Fig. 2).

Ligand hfs

The interaction between the delocalized unpaired electron and the magnetic moment of the nuclei surrounding the central ion can give rise to extra hfs lines in the ESR spectrum. Once again, only the isotropic parts are observable in a solution. Thus, in our model only the S 3*s* part of the wave function of the unpaired electron contributes to the hfs. The interaction energy becomes

$$a_S = \left(\frac{\alpha'}{2}\right)^2 (1-n^2)\, 2\, g_I'\beta_0\beta_N \frac{8\pi}{3}\delta_{3s}(r).$$

g_I' is the nuclear g-factor of the S nucleus, and $\delta_{3s}(r)$ is the $3s$ electron density at the S nucleus. Applying a procedure suggested by Löwdin [28], in which available Hartree wave functions are compared to hydrogen-like wave functions, we get $Z_{\text{eff}} = 8.46$ for determination of $\delta_{3s}(r)$ of S^{2-} (cf. Table IX of [28]). In our case S is more like a neutral atom, and therefore we choose $Z_{\text{eff}} = 9$ (cf. [26]), which gives $\delta_{3s}(r) = 8.6$ a. u.

Techniques and results

The dithiocarbamates (kindly supplied to us by fil. lic. S. Åkerström) were mostly studied in benzene solution (for preparations, see [8]). The solutions were carefully deoxygenated, as dissolved oxygen broadens the resonance lines considerably. The ESR spectra were recorded with a Varian V-4500 spectrometer operating at a frequency close to 9500 Mc/s. The unusually small line width of the solutions is apparent from Fig. 2. Prof. J. E. Wertz (Univ. of Minnesota) has suggested to us that the Cu compound could therefore be used as a standard for determination of free radical ESR intensities.

g-values and hfs constants

In a previous paper [9] preliminary values of g_s and a have been reported. g_s-values of Cu dithiocarbamates with different substituents are given in Table 2.

Table 2. g_s-values of Cu dithiocarbamates with different substituents $R'R''$.

Solvent: C_6H_6. Estimated accuracy: ± 0.0003.

$R'R''$	$CH_3C_6H_5$	$(CH_3)_2$	$(C_2H_5)_2$	$(iso\text{-}C_3H_7)_2$
g_s	2.0435	2.0448	2.0453	2.0437

All compounds listed there have $a = (0.74 \pm 0.02)\ 10^{-2}$ cm $^{-1}$. The only investigated Ag compound ,$[(iso\text{-}C_3H_7)_2NCS_2]_2$ Ag, has $g_s = 2.0194 \pm 0.0003$ and $a = -(0.280 \pm 0.002)\ 10^{-2}$ cm $^{-1}$. In order to determine $g_{\|}$, $g_{\perp}$, A and B for Cu, attempts have been made to make diluted single crystals, but unfortunately the crystals obtained were too small. From the rather complicated spectrum of a polycrystalline sample of the diluted crystals (Fig. 3) we estimate the parameters for the $iso\text{-}C_3H_7$ Cu compound to be:

$$g_{\|} = 2.087 \pm 0.007,\ g_{\perp} = 2.023 \pm 0.007,\ A = -(1.54 \pm 0.10)\ 10^{-2}\ \text{cm}^{-1},$$
$$B = -(0.35 \pm 0.05)\ 10^{-2}\ \text{cm}^{-1}.$$

The Ag compounds cannot be isolated, but from spectra of the frozen solutions we get:

$$g_{\|} = 2.035 \pm 0.003,\ g_{\perp} = 2.011 \pm 0.003,\ A = -(0.37 \pm 0.02)\ 10^{-2}\ \text{cm}^{-1},$$
$$B = -(0.24 \pm 0.02)\ 10^{-2}\ \text{cm}^{-1}.$$

All g-values are corrected for second order terms (cf. equation (2)).

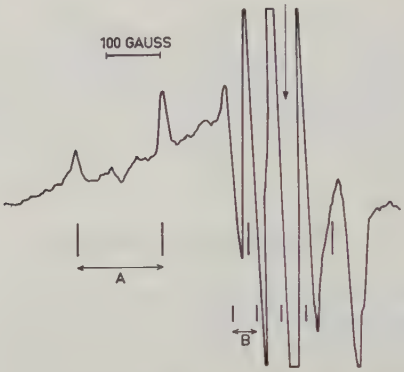


Fig. 3. ESR spectrum of a polycrystalline sample of $[(iso\text{-}C_3H_7)_2NCS_2]_2Cu$ diluted with the corresponding Ni compound.

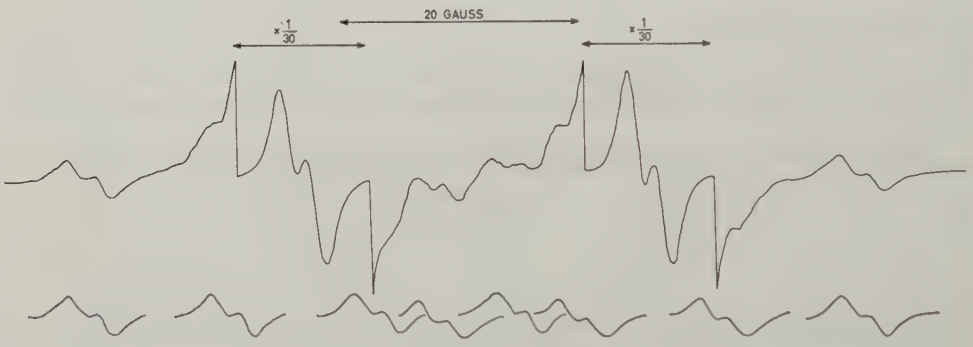


Fig. 4. Upper part: ESR spectrum of $[(iso\text{-}C_3H_7)_2NCS_2]_2Ag$ in benzene. Amplification at main peaks reduced 30 times as indicated. Lower part: S^{33} hyperfine lines, constructed by reproducing the shapes of main peaks with theoretical amplitudes and with spacing to fit experimental spectrum.

S^{33} hfs

Of the naturally abundant S isotopes, only S^{33} with abundance 0.74 % has a nonvanishing nuclear spin (3/2) and magnetic dipole moment (0.643 nuclear magnetons). If the 4 S atoms surrounding the dithiocarbamate central ion are equivalent, each of the Cu or Ag hyperfine lines will have $(2I_{S^{33}}+1)=4$ satellites. The possibility that more than one of the S atoms are S^{33} can be neglected. The upper part of Fig. 4 shows the spectrum of $[(iso\text{-}C_3H_7)_2NCS_2]_2$ Ag in benzene, and the lower part of the same figure shows the S^{33} hyperfine structure with theoretical amplitudes and with an assumed hyperfine splitting of 11.9 gauss ($1.12\cdot10^{-3}$ cm^{-1}). The agreement with experimental amplitudes is good. For the Cu compounds, the same extra hfs is observed with a splitting of 11.2 gauss ($1.07\cdot10^{-3}$ cm^{-1}). The resolution is, however, not as good as for Ag due to the greater line width of the Cu resonance lines.

Optical absorption spectra

The absorption maxima in visible light have been determined by Åkerström [8]. The Cu compounds all show a maximum around 23 000 cm^{-1} and the Ag compouds around 16 000 cm^{-1}. We have measured the absorption in u.v. (with a Beckman DU spectrophotometer) and have found maxima at ca. 33 600 cm^{-1} and 34 000 cm^{-1} for Cu and Ag respectively. In order to get some guidance in the assignment of the transitions, we have studied the solvent effect on the maxima, as one might expect Δ_2 to be more solvent dependent than Δ_1. Using the solvents C_6H_6, CCl_4 and $CHCl_3$ we find a shift of 160 cm^{-1} for the Cu maximum in the visible and 880 cm^{-1} for the Cu maximum in u.v. These results suggest that the absorption maximum in the visible is Δ_1 and the u.v. transition Δ_2.

Bonding parameters

Using the equations given in the theoretical part the following values of $\varkappa$, $1-n^2$ and the bonding parameters are obtained:

	Cu	Ag
$\varkappa$	0.27	0.48
$1-n^2$	0.053	0.052
α^2	0.53	0.46
β_1^2	0.85	0.54
β^2	0.98	0.76

Discussion

In the treatment given leading up to the bonding parameters quite a few assumptions and approximations have been made, which must be kept in mind when conclusions are drawn from the results obtained. Some remarks of a qualitative nature will, however, be made.

$\varkappa$

$\varkappa$, representing the isotropic hyperfine coupling with the central nucleus, seems to be the parameter which is most insensitive to the detailed assumptions about wave functions etc. There are some indications that for a given ion $\varkappa$ is independent of the bonding [25], but our value for Cu differs considerably from the value obtained by Maki and McGarvey [2] for Cu(II) bis-acetylacetonate (0.33). On the other hand, $\varkappa$ is not proportional to the unpaired electron density (α^2) at the central ion. If the intermixing with a configuration $ns(n+1)s$ ($n=3$ for Cu) were responsible for the isotropic hfs [25], such a mechanism would probably be more important for higher n-values, as the exitation energy becomes less. This is supported by the experimental data. Using formula (1) and neglecting the smaller terms, we get

$$\varkappa_{Cu}=0.26,\ \varkappa_{Ag}=0.40,\ \varkappa_{Au}=0.72.$$

g_s and a for Au is taken from [9]. The trend is quite clear. It might be mentioned that Heine [18] has given an explanation of the isotropic hfs in terms of

exchange effects and that he gets the right order of magnitude. A correlation of his theory with the results obtained above has not been tried.

Hybridization

The hybrid parameter n turns out to be very similar for Cu and Ag. The valency angle between equivalent hybrids would be 93.2° and 93.1° respectively. Our results seem to indicate, that the bonds do not have much s character, but the similarity between the Cu and Ag n-values is probably only accidental, as replacement of Cu(II) with Ag(II) very likely would introduce larger changes in the geometrical arrangement. It should also be remembered that we have not considered the possibility of promotion of an electron to the $4s$ orbital of S, which would give rise to a large isotropic hfs.

Covalency of bonds

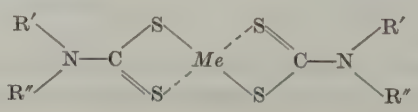
The parameters α, β_1 and β are related to the degree of covalency of the bonds. Their squares are usually thought to range from 1 (pure ionic bonds) to 0.5 (pure covalent bonds), but it does not seem to have been proved that they cannot be less than 0.5 [29]. In our case no parameter is significantly less than 0.5.

We will first discuss the bonding situation in Cu. Our results indicate that the σ bonds (characterized by α^2) are almost pure covalent, and the degree of covalency is much higher than in the investigated cases of coordination to O or N [2, 3]. The covalency of the in-plane π bonding (β_1^2) is not so strong and is similar for S, O and N. Similarity is also found in the out-of-plane π bonding (β^2), which in all cases has little covalent character. The interaction with the ligand π electron system is thus very small, which is somewhat surprising in view of the effect (probably transmitted via the π electron system) of the alkyl substituents on the stability of the complexes [8]. On the other hand, the substituents do not much influence the magnetic properties of the complexes as is evident from Table 2. The differences in g-values are small, and no simple correlation can be made between the stabilities and g-values.

Qualitatively, the results for Ag are similar to those for Cu, but the actual values of the bonding parameters are less reliable. For example, it is quite possible that the transition corresponding to Δ_2 is not identical to the observed transition at 34 000 cm^{-1} but that it has higher energy. Precise determination of the bonding parameters is also difficult due to the inaccuracy of the experimental g-values. However, the bonding parameters are smaller than for Cu, which is in accordance with the well-known fact that $4d$ elements usually form stronger covalent bonds than $3d$ elements. π bonding thus seems to be of greater importance for Ag than for Cu.

SUMMARY

Electron spin resonance experiments have been performed on the *N*,*N*-dialkyldithiocarbamates of Cu(II) and Ag(II)



The hyperfine structure of the naturally abundant S^{33} has been detected. The constant $\varkappa$, introduced to account for isotropic hyperfine structure, has been estimated for Cu(II), Ag(II) and Au(II), and is found to increase in the same order. Parameters related to the degree of covalency of the Cu and Ag bonding have been calculated. They indicate that the sulphur hybrid directed towards the central ion has only little *s* character, and that the metal-sulphur σ bond is quite covalent.

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Beiträge zur Chemie α-substituierter Sulfide. III*

Über in 3-Stellung aromatisch substituierte 4-Thiaisochroman-1-one

Von Alexander Senning und Sven-Olov Lawesson

Über das 4-Thiaisochroman-1-on (1,3-Benzthioxan-4-on) und seine Derivate ist bisher nur wenig gearbeitet worden. Kaufmann [1], Chandra [2], Mowry [3, 4, 5] und Böhme [6] beschrieben eine Anzahl von Derivaten, während wir selbst vor einiger Zeit das 3,3-Dichlorderivat sowie den Grundkörper darstellen konnten [7]. Von den Methoden der erstgenannten Autoren ist wohl nur die von Mowry [4] von allgemeinerem präparativen Interesse. Sie beruht auf der schwefelsäurekatalysierten Umsetzung der Diazetate aromatischer Aldehyde mit Thiosalizylsäure in Essigsäure.

Während Mowry, dessen Interesse hauptsächlich den Kondensationsreaktionen der Salizylsäure galt, vermutlich aus Analogiegründen annahm, dass die Umsetzung von Thiosalizylsäure mit den freien aromatischen Aldehyden in erster Linie zu Triphenylmethanderivaten führen würde und keine diesbezüglichen Versuche durchführte, konnten wir zeigen, dass aromatische Aldehyde analog dem Formaldehyd mit Thiosalizylsäure in siedendem Eisessig in Gegenwart von Chlorwasserstoff teils 4-Thiaisochroman-1-onderivate und teils substituierte Benzal-bis-(*o*-karboxyphenylsulfide) bilden. Das primär gebildete Halbmerkaptal I reagiert teils unter Ringschluss zum Lakton II, teils mit einem weiteren Molekül Thiosalizylsäure zum Merkaptal III.

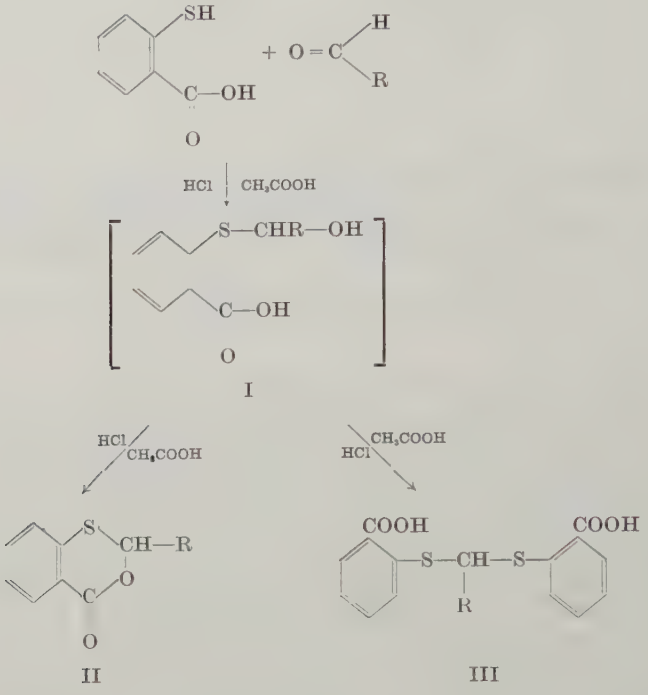
Über die bei dieser Reaktion gebildeten Dikarbonsäuren, die wir, da sie auf Grund ihrer Konstitution potentielle Fungizide darstellen, auf ihre biologische Aktivität zu prüfen gedenken, werden wir zu einem späteren Zeitpunkt berichten. Dass die von uns erhaltenen Säuren Merkaptale und nicht die mit ihnen isomeren Triphenylmethanderivate sind, konnten wir aus der Abwesenheit freier Merkaptangruppen schliessen. Benzal-bis-(*o*-karboxyphenylsulfid) (III, $R = C_6H_5$) z. B. entfärbt methanolische Jodlösung im Gegensatz zu Thiosalizylsäure nicht und ebenso fehlt im Infrarotspektrum die SH-Bande bei 3,98 μ.

Bei der Umsetzung mit Furfurol wurde kein 4-Thiaisochroman-1-on-derivat erhalten, da während der Reaktion starke Verkohlung stattfand. Das 2-Thienylderivat konnte nur in sehr geringer Ausbeute dargestellt werden.

Die Karbonylbande der hier beschriebenen Verbindungen liegt durchweg bei 5,80 μ, also der für δ-Laktone normalen Frequenz, im Gegensatz zum 3,3-Dichlor-4-thiaisochroman-1-on, bei dem die Karbonylbande charakteristisch verschoben war [7]. Vgl. auch [8].

Zur Zeit beschäftigen wir uns mit der Kondensation aliphatischer Aldehyde mit Thiosalizylsäure, während wir gleichzeitig die Möglichkeit untersuchen, Ketone zu dieser Reaktion heranzuziehen.

* II. Mitteilung siehe Literaturzitat [7].



Beschreibung der Versuche

Alle Schmelzpunkte sind unkorrigiert. Die Infrarotspektrogramme wurden mit einem Spektrographen Perkin-Elmer Modell 137 aufgenommen. Die Analysen wurden im Institut für Analytische Chemie der Universität Uppsala ausgeführt.

Die Thiosalizylsäure wurde von der Firma Schuchardt, München bezogen und schmolz zwischen 161° und 164° (Literatur: F: 164°).

Versuchsanordnung

In einem Kolben mit Gaseinleitungsrohr und Rückflusskühler mit aufgesetztem Chlorkalziumrohr wurden 0,15 bis 0.30 Mol Thiosalizylsäure und eine äquivalente Menge Aldehyd in 300 bis 500 ml Eisessig gelöst und unter Rückflusskochen 3 Stunden lang trockener Chlorwasserstoff eingeleitet. Nach Beendigung der Reaktion wurde der grösste Teil des Eisessigs abdestilliert, das Ausgefallene abfiltriert und mit verdünnter Sodalösung digeriert. Das Filtrat wurde in einen Überschuss kaltes Wasser gegossen, die Fällung abfiltriert und ebenfalls mit verdünnter Sodalösung digeriert. Die unlöslichen Anteile wurden vereinigt und zur Analyse umkristallisiert. Aus den mit Äther extrahierten vereinigten alkalischen Lösungen wurde die Säure mit konzentrierter Salzsäure ausgefällt.

3-Phenyl-4-thiaisochroman-1-on

Ausgangsmaterial: 46,2 g (0,3 Mol) Thiosalizylsäure und 31,8 g (0,3 Mol) Benzaldehyd.

Rohausbeute 11,5 g = 24 % der Theorie. (F: 86°–88°.) Nach mehrmaligem Umkristallisieren aus leichtsiedendem Petroläther schmolz das 3-Phenyl-4-thiaisochroman-1-on (farblose Säulen) zwischen 83° und 84° (Literatur: F: 90°). Unser Produkt hatte folgende Analysendaten: ($C_{14}H_{10}O_2S$) gef. C 69,52 %; H 4,21 %; S 13,20 %; ber. C 69,40 %; H 4,16 %; S 13,23 %. Mowry [4] fand für sein Produkt C 69,4 %; H 4,43 %. Er gibt keinen Wert für Schwefel an.

3-(3,4-Methylendioxyphenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 46,2 g (0,3 Mol) Thiosalizylsäure und 45,0 g (0,3 Mol) Piperonal (F: 37°, Literatur: F: 37°).

Rohausbeute: 12,8 g = 15 % der Theorie (F: 118°–121°). Nach mehrmaligem Umkristallisieren aus Essigester und leichtsiedendem Petroläther schmolz die Substanz (farblose grobe Nadeln) zwischen 130° und 131° (Literatur: F: 129° [4]).

3-(2-Chlorphenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 23,1 g (0,15 Mol) Thiosalizylsäure und 21,0 g (0,15 Mol) 2-Chlorbenzaldehyd ($n_D^{20} = 1.5677$; Literatur: $n_D^{19,7} = 1,5673$; Firma British Drug House).

Rohausbeute: 11,0 g = 27 % der Theorie (F: 145°–155°). Nach mehrmaligem Umkristallisieren aus Essigester schmolz das 3-(2-Chlorphenyl)-4-thiaisochroman-1-on (farblose derbe sechsseitige Prismen) zwischen 155° und 157°.

$C_{14}H_9ClO_2S$	gef. S 11,46 %;	Cl 12,81 %
	ber. S 11,59 %;	Cl 12,81 %

3-(4-Chlorphenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 23,1 g (0,15 Mol) Thiosalizylsäure und 21,0 g (0,15 Mol) 4-Chlorbenzaldehyd (F: 45°–48°, Literatur: F: 47,5°; Firma Kahlbaum).

Rohausbeute: 9,2 g = 22 % der Theorie (F: 97°–125°). Nach mehrmaligem Umkristallisieren aus Äther schmolz das 3-(4-Chlorphenyl)-4-thiaisochroman-1-on (farblose derbe vierseitige Prismen) zwischen 116° und 117°.

$C_{14}H_9ClO_2S$	gef. S 11,49 %;	Cl 12,80 %
	ber. S 11,59 %;	Cl 12,81 %

3-(2-Nitrophenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 23,1 g (0,15 Mol) Thiosalizylsäure und 22,6 g (0,15 Mol) 2-Nitrobenzaldehyd (F: 44°, Literatur: F: 44°; Firma Merck).

Rohausbeute: 8,4 g = 20 % der Theorie (F: 170°–179°). Nach mehrmaligem Umkristallisieren aus Essigester schmolz das 3-(2-Nitrophenyl)-4-thiaisochroman-1-on (farblose derbe sechsseitige Prismen) zwischen 177° und 179°.

$C_{14}H_9NO_4S$	gef. S 11,02 %;	N 4,85 %
	ber. S 11,16 %;	N 4,88 %

3-(3-Nitrophenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 23,1 g (0,15 Mol) Thiosalizylsäure und 22,6 g (0,15 Mol) 3-Nitrobenzaldehyd (F: 53°–56°, Literatur: F: 58°; Firma Kahlbaum).

Rohausbeute: 18,6 g = 43 % der Theorie (F: 120°–140°). Nach mehrmaligem Umkristallisieren aus Essigester schmolz das 3-(3-Nitrophenyl)-4-thiaisochroman-1-on zwischen 139° und 141° (farblose mikrokristalline Agglomerate).

$C_{14}H_9NO_4S$	gef. S 11,03 %;	N 4,83 %
	ber. S 11,16 %;	N 4,88 %

3-(4-Nitrophenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 23,1 g (0,15 Mol) Thiosalizylsäure und 22,6 g (0,15 Mol) 4-Nitrobenzaldehyd (F: 103°–106,5°, Literatur: F: 106,5°; Firma Kahlbaum). Rohausbeute: 26,5 g = 62 % der Theorie (F: 193°–205°). Nach mehrmaligem Umkristallisieren aus Essigester schmolz das 3-(4-Nitrophenyl)-4-thiaisochroman-1-on (farblose derbe Nadeln) zwischen 212° und 213°.

$C_{14}H_9NO_4S$	gef. S 11,05 %;	N 4,84 %
	ber. S 11,16 %;	N 4,88 %

3-(2-Thienyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 46,2 g (0,3 Mol) Thiosalizylsäure und 33,6 g (0,3 Mol) frisch destillierter 2-Thiophenaldehyd ($n_D^{20} = 1{,}5917$; Literatur: $n_D^{20} = 1{,}5920$).

Rohausbeute 1,6 g = 2 % der Theorie (F: 78°–86°). Nach mehrmaligem Umkristallisieren aus Äthanol schmolz das 3-(2-Thienyl)-4-thiaisochroman-1-on (farblose derbe quadratische Tafeln) zwischen 87° und 88°. Es war von einer schwer zu entfernenden bräunlichen Verunreinigung begleitet.

$C_{12}H_8O_2S_2$	gef. C 57,79 %;	H 3,22 %;	S 25,85 %
	ber. C 58,04 %;	H 3,25 %;	S 25,83 %

ZUSAMMENFASSUNG

Die Bildung des 4-Thiaisochroman-1-on-systems durch Kondensation von Thiosalizylsäure mit Formaldehyd [7] gelingt auch mit aromatischen Aldehyden. Die Umsetzung von Thiosalizylsäure mit acht aromatischen Aldehyden lieferte sechs neue und zwei bekannte 4-Thiaisochroman-1-on-derivate.

Wir sind Herrn Professor A. Fredga für die Überlassung des Arbeitsplatzes und Jordbrukets Forskningsråd für die finanzielle Unterstützung unserer Arbeit zu Dank verpflichtet.

Laboratorium für Organische Chemie, Chemisches Institut der Universität Uppsala, Uppsala, Schweden.

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Studies on synthetic growth substances

XIII. Optically active 2- and 3-chlorophenoxy-propionic acids and their absolute configuration

By Arne Fredga, Anne-Marie Weidler and Christina Grönwall

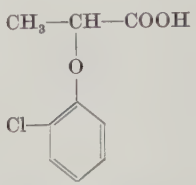
With 6 figures in the text

In the course of current investigations on optically active plant growth regulators, Matell, Fredga and co-workers [1, 2, 3, 4, 5, 6] have prepared and resolved a number of chlorosubstituted phenoxy-propionic acids, among them the six dichloro acids. From studies on the corresponding phenoxy-acetic acids, it is well known that the positions of the chlorine atoms are essential for the biological activity [7, 8]. In the case of the propionic acids (and their higher homologues) there is also a more or less pronounced difference in biological activity between the D- and L-forms, the former having the greater biological effect. For instance, the D-(+)-4-chlorophenoxy-propionic acid is an auxin of intermediate activity, while the L-(−)-acid is about 100 times less active.

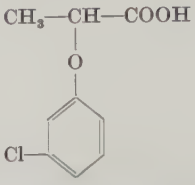
The relative configurations of the acids, including the unsubstituted phenoxy-propionic acid, have been studied by the quasi-racemate method, recently reviewed by Fredga [9]. The phenoxy-propionic acid could be correlated to the lactic acid by purely chemical methods [10, 11], and thus the absolute configuration of the growth regulators was established. As a matter of fact, the various aryloxy-propionic acids and their homologues constitute a very interesting material for studying the applicability and the limitations of the quasi-racemate method. These questions will be discussed elsewhere.

From stereochemical as well as from plant physiological points of view it seemed of interest to extend the investigations to the 2- and 3-chlorophenoxy-propionic acids (I and II).

The racemic acids have been described by previous authors [12, 13]. The laevorotatory forms can in both cases be isolated via the strychnine salts, but none of the common bases was found quite satisfactory for isolating the (+)-acids. It was found, however, that the optically active acids are easily soluble in carbon tetrachloride at room temperature, while the racemic acids are only sparingly soluble. Dextrorotatory acids of high activity could accordingly be obtained by rapid extraction of the acids isolated from the mother liquors from the strychnine salts. The 2-acid could be brought to maximum activity by recrystallisation from dilute formic acid. The 3-acid was purified via the (−)-phenyl-isopropylamine salt.



(I)



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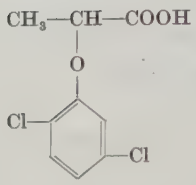

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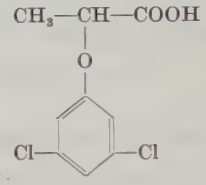

(IV)

Table 1. $[M]_D^{25}$ for the (−)-chlorophenoxy-propionic acids in various media.

	2-chloro	3-chloro	4-chloro
Acetone	−60.4°	−109.1°	−115.1°
Ethanol (abs.)	−60.7°	−78.3°	−82.2°
Ethanol (96 %)	−52.7°	−71.7°	–
Chloroform	−29.0°	−48.8°	−47.0°
Benzene	+38.8°	+2.7°	+22.2°
Water	+48.8°	−21.0°	+36.6°

The molecular rotations of the (−)-acids in various solvents are given in Table 1. For comparison, the values for the 4-chloro derivative reported by Matell [2] are included in the table (his measurements actually refer to the (+)-acid and are here given with opposite signs). It is noteworthy that the rotatory power in common 96 % alcohol is about 10 % lower than in absolute ethanol.

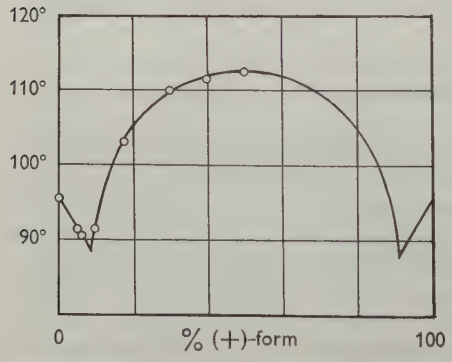

Fig. 1. (+)- and (−)-2-chlorophenoxy-propionic acid (I).

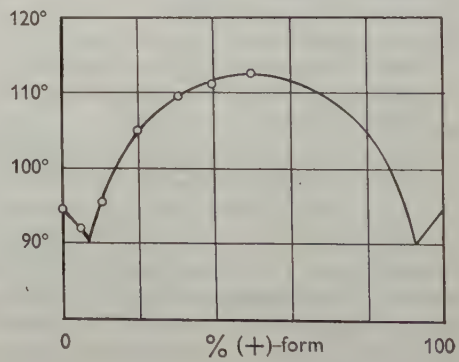

Fig. 2. (+)- and (−)-3-chlorophenoxy-propionic acid (II).

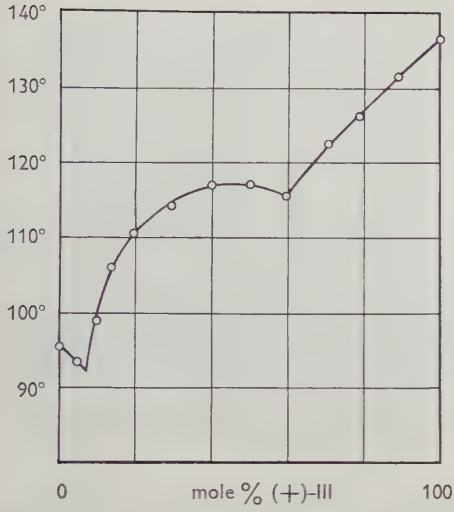

Fig. 3. (−)-2-chlorophenoxy-propionic acid (I) and (+)-2,5-dichlorophenoxy-propionic acid (III).

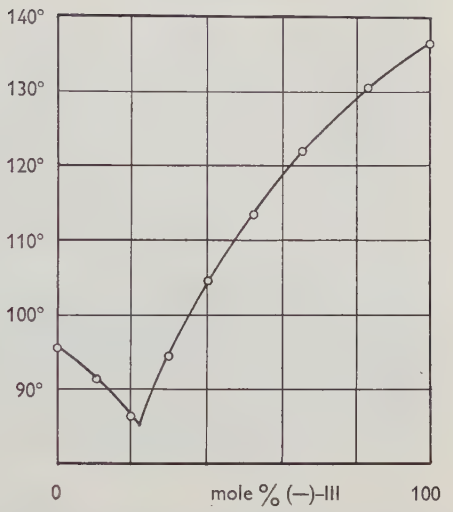

Fig. 4. (−)-2-chlorophenoxy-propionic acid (I) and (−)-2,5-dichlorophenoxy-propionic acid (III).

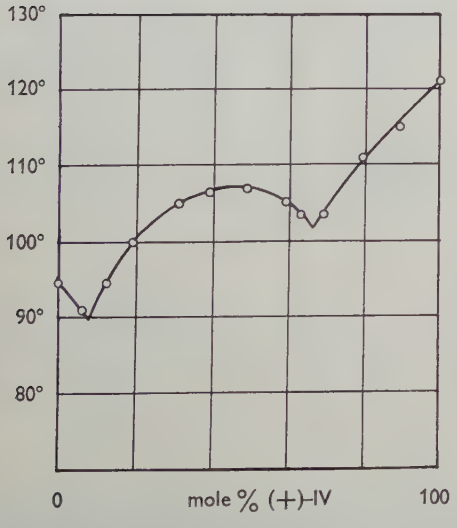

Fig. 5. (−)-3-chlorophenoxy-propionic acid (II) and (+)-3,5-dichlorophenoxy-propionic acid (IV).

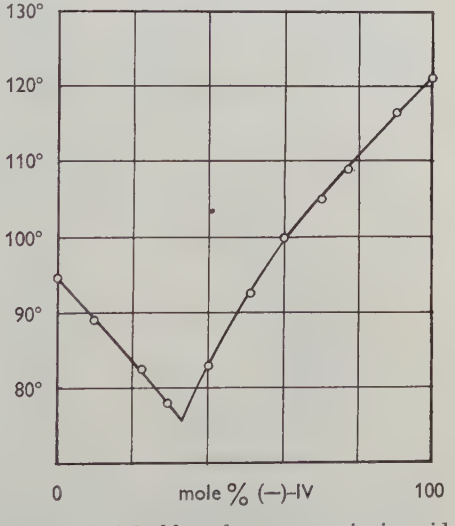

Fig. 6. (−)-3-chlorophenoxy-propionic acid (II) and (−)-3,5-dichlorophenoxy-propionic acid (IV).

The absolute configurations could be determined by the quasi-racemate method [9]. The inactive acids are in both cases true racemic compounds with higher melting-points than the corresponding enantiomorphs (Figs. 1 and 2). The (−)-2-chlorophenoxy-propionic acid formed a typical quasi-racemate with the (+)-2,5-dichlorophenoxy acid (III), while the two laevorotatory acids give a diagram of the common eutectic type (Figs. 3 and 4). Thus the acids with the same mode of rotation have the same configuration. In the same way, the 3-chlorophenoxy-propionic acid could be related to the 3,5-dichlorophenoxy-propionic acid (IV) with the same mode of rotation (Figs. 5 and 6). As the dextrorotatory dichloro acids have D-configuration [5], the same holds good for the 2- and 3-monochloro derivatives. The D-configuration of the dextrorotatory 4-chlorophenoxy-propionic acid has already been established by Matell [2]. The data for the optical activity of the three acids (Table 1), which show an obvious parallelism, are in accordance with this result.

The auxin activity of the acids is being investigated by Professor B. Åberg of the Royal Agricultural College.

Experimental

The racemic acids were prepared from the corresponding phenols (commercial samples) and ethyl α-bromopropionate following conventional methods. They were recrystallized from dilute formic acid.

Resolution of the 2-chlorophenoxy-propionic acid (Experiments by A.-M. Weidler). Preliminary experiments were performed with the common alkaloids. Acid (0.001 mole) and base (0.001 mole) were dissolved in small volumes of solvent, and the salt obtained was filtered off and dried. The acid was then liberated and the rotatory power determined in common 96 % ethanol (Table 2). Both strychnine and cinchonidine give high activity and of these the former was selected for the resolution on a preparative scale.

Table 2.

Base	Solvent	Yield of salt, %	$[\alpha]_D^{25}$
Strychnine	Ethanol (33 %)	40.5	−20.5°
Cinchonidine	Ethanol (50 %)	29.5	−23.5°
Cinchonine	Ethanol (50 %)	46.5	−9.5°
Quinine	Ethanol (40 %)	79.5	−1.5°
Quinine	Acetone (40 %)	39	−8.5°
Brucine	Ethanol (50 %)	42.5	−4.5°
(−)-α-Phenyl-isopropylamine	Water	38	+1.5°

24.0 g (0.12 mole) acid and 40 g (0.12 mole) strychnine were dissolved in a mixture of 120 ml 96 % ethanol and 200 ml water. The salt was filtered off after 20 hours and recrystallised three times from dilute ethanol. After each crystallisation, a small sample of the salt was decomposed and the activity of the acid determined in 96 % ethanol. The results are given in Table 3; the (−)-acid is practically pure already in the first crystallisation.

Table 3.

Crystallisation	1	2	3	4
ml ethanol	120	50	20	20
ml water	200	100	40	40
g salt obtained	21.0	18.8	16.5	15.5
$[\alpha]_D^{25}$ of the acid	−25.5°	−26°	−25.5°	−25°

(−)-2-Chlorophenoxy-propionic acid. 14.6 g of the strychnine salt was decomposed with 120 ml of 2-*M* sodium carbonate solution and the strychnine removed by repeated extractions with chloroform. After acidification with sulphuric acid in excess, the 2-chloro acid was extracted with ether. The yield of crude product was 4.95 g; after two recrystallisations from cyclohexane 4.2 g remained. The acid forms very thin glistening needles with m.p. 95–95.5°

$C_9H_9O_3Cl$ (200.62) Cl calc. 17.67; found 17.69.

0.2036 g acid dissolved in *acetone* to 10.00 ml: $\alpha_D^{25} = -1.225°$ (2 dm). $[\alpha]_D^{25} = -30.1°$; $[M]_D^{25} = -60.4°$. 0.2032 g acid dissolved in *abs. ethanol* to 10.00 ml: $\alpha_D^{25} = -1.23°$ (2 dm). $[\alpha]_D^{25} = -30.3°$; $[M]_D^{25} = -60.7°$. 0.2075 g acid dissolved in *96 % ethanol* to 10.00 ml: $\alpha_D^{25} = -1.09°$ (2 dm). $[\alpha]_D^{25} = -26.3°$; $]M]_D^{25} = -52.7°$. 0.2038 g acid dissolved in *chloroform to* 10.00 ml: $\alpha_D^{25} = -0.59°$ (2 dm). $[\alpha]_D^{25} = -14.5°$; $[M]_D^{25} = -29.0°$. 0.2027 g acid dissolved in *benzene* to 10.00 ml: $\alpha_D^{25} = -0.785°$ (2 dm). $[\alpha]_D^{25} = -19.4°$; $[M]_D^{25} = +38.8°$. 0.2045 g acid *neutralised* and dissolved in *water* to 10.00 ml: $\alpha_D^{25} = +0.995°$ (2 dm). $[\alpha]_D^{25} = +24.3°$; $[M]_D^{25} = +48.8°$.

(+)-2-Chlorophenoxy-propionic acid. The mother liquor from the first crystallisation of the strychnine salt yielded 10 g acid having $[\alpha]_D \sim +10°$ (abs. ethanol). This was powdered and digested with two 40 ml portions of cold carbon tetrachloride. The acid obtained from the extracts (3.8 g) was recrystallised twice from cyclohexane, yielding 3.2 g with $[\alpha]_D^{25} = +27.5°$ (acetone). Further recrystallisations from the solvent had no effect. 2.3 g of the acid were therefore recrystallised from dilute formic acid until maximum activity was obtained (Table 4).

Table 4.

Crystallisation	1	2	3	4
g acid	2.0	–	1.4	1.2
$[\alpha]_D^{25}$ in acetone	+28.3°	+29.7°	+30.2°	+30.1°

The acid forms very thin, glistening needles, and is quite similar to the (−)-form. M.p. 95–95.5°

$C_9H_9O_3Cl$ (200.62) Cl calc. 17.67; found 17.69.

0.2036 g acid (3rd crystallisation) dissolved in *acetone* to 10.00 ml: $\alpha_D^{25} = +1.23°$ (2 dm). $[\alpha]_D^{25} = +30.2°$; $[M]_D^{25} = +60.6°$. 0.2086 g acid (4th crystallisation) dissolved in *acetone* to 10.00 ml: $\alpha_D^{25} = +1.255°$ (2 dm). $[\alpha]_D^{25} = +30.1°$; $[M]_D^{25} = +60.3°$.

Resolution of the 3-chlorophenoxy-propionic acid (Experiments by C. Grönwall). Preliminary experiments were carried out as described above. As seen from Table 5, strychnine gives by far the best resolution. (−)-Phenyl-isopropylamine (benzedrine) could be used for purification of the (+)-form.

Table 5.

Base	Solvent	Yield of salt, %	$[\alpha]_D^{25}$
Strychnine	Ethanol (50 %)	56	−35°
Strychnine	Ethanol (60 %)	30	−39°
Cinchonidine	Ethanol (50 %)	70	+2°
Quinine	Ethanol (50 %)	76	−10°
(−)-α-Phenyl-isopropylamine	Ethanol (15 %)	52	+9°

30 g (0.15 mole) acid and 47 g (0.15 mole) strychnine were dissolved together in 225 ml of hot 96 % ethanol, and 225 ml of hot water were added. The salt was filtered off after 8 hours and recrystallised until maximum activity. The course of the resolution was followed as described above (Table 6).

Table 6.

Crystallisation	1	2	3	4	5
ml ethanol	225	100	70	60	60
ml water	225	150	105	90	90
g salt obtained	24.1	18.7	16.4	13.8	12.3
$[\alpha]_D^{25}$ of the acid	−32°	−36.5°	−35.5°	−36°	−36°

The mother liquor from the first crystallisation was left in a refrigerator overnight. 9.8 g Salt separated and was recrystallised utilising the successive mother liquors from the previous experiment. In this way an additional crop of 9.4 g pure (−)-salt was obtained.

(−)-3-Chlorophenoxy-propionic acid. The total amount of pure strychnine salt (20.8 g) was decomposed and the acid liberated as described above for the 2-chloro acid. The yield of crude product was 6.6 g; after two recrystallisations from dilute formic acid 6.4 g remained. The acid forms glistening needles with m.p. 93.5–94.5°.

$C_9H_9O_3Cl$ (200.62) Cl calc. 17.67; found 17.54.

0.2050 g acid dissolved in *acetone* to 10.00 ml: $\alpha_D^{25} = -2.23°$ (2 dm). $[\alpha]_D^{25} = -54.4°$; $[M]_D^{25} = -109.1°$. 0.2038 g acid dissolved in *abs. ethanol* to 10.00 ml: $\alpha_D^{25} = -1.59°$ (2 dm). $[\alpha]_D^{25} = -39.0°$; $[M]_D^{25} = -78.3°$. 0.2093 g acid dissolved in *96 % ethanol* to 10.00 ml: $\alpha_D^{25} = -1.495°$ (2 dm). $[\alpha]_D^{25} = -35.7°$; $[M]_D^{25} = -71.7°$. 0.2044 g acid dissolved in *chloroform* to 10.00 ml: $\alpha_D^{25} = -0.995°$ (2 dm). $\alpha_D^{25} = -24.3°$; $[M]_D^{25} = -48.8°$. 0.2023 g acid dissolved in *benzene* to 10.00 ml: $\alpha_D^{25} = +0.055°$ (2 dm); $[\alpha]_D^{25} = +1.4°$; $[M]_D^{25} = +2.7°$. 0.2058 g acid *neutralised* and dissolved in *water* to 10.00 ml: $\alpha_D^{25} = -0.43°$ (2 dm). $[\alpha]_D^{25} = -10.4$; $[M]_D^{25} = -21.0°$.

(+)-3-Chlorophenoxy-propionic acid. On evaporation, the mother liquors from the first crystallisation of the strychnine salt gave several salt fractions, from which in all 13.5 g more or less dextrorotatory acid was isolated. Three extractions with 40 ml portions of cold carbon tetrachloride yielded 8.2 g acid having $[\alpha]_D^{25} \sim +50^\circ$ (acetone). On recrystallisation from dilute formic acid, the rotatory power increased very slowly. In this respect there seems to be an obvious difference between the 2-chloro and the 3-chloro acids.

5.0 g (0.025 mole) of the strongly dextrorotatory 3-acid were therefore dissolved with 3.3 g (0.025 mole) of (−)-phenyl-isopropylamine in 40 ml water + 8 ml ethanol. The salt obtained (fine needles) was recrystallised from 30 ml water + 6 ml ethanol. On decomposition it yielded 3.4 g acid. After recrystallisation from dilute formic acid 3.2 g of glistening needles, quite similar to the (−)-form were obtained. M.p. 93.5–94.5°.

$C_9H_9O_3Cl$ (200.62) Cl calc. 17.67; found 17.57.

0.2061 g acid dissolved in *acetone* to 10.00 ml: $\alpha_D^{25} = +2.245^\circ$ (2 dm). $[\alpha]_D^{25} = -54.5^\circ$; $[M]_D^{25} = +109.3^\circ$.

Melting-point diagrams. The samples were prepared by dissolving weighed quantities of the components in a few drops of acetone. After evaporation of the solvent, the residue was powdered and dried. The melting-points were determined in a Kofler hot stage microscope [14].

SUMMARY

The 2- and 3-chlorophenoxy-propionic acids have been resolved into the optically active forms. The 2-acid gave $[\alpha]_D^{25} = +30.2^\circ$ and -30.1° respectively in acetone solution. The corresponding values for the 3-acid were $[\alpha]_D^{25} = +54.5^\circ$ and -54.4°. Molecular rotations in various solvents are given in Table 1.

The dextrorotatory acids are sterically related to the D-(−)-lactic acid. The growth-regulating effect of the optically active acids is being investigated.

ACKNOWLEDGEMENTS

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ARKIV FÖR KEMI Band 17 nr 24

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A convenient synthesis of inactive selenocystine

By Göran Zdansky

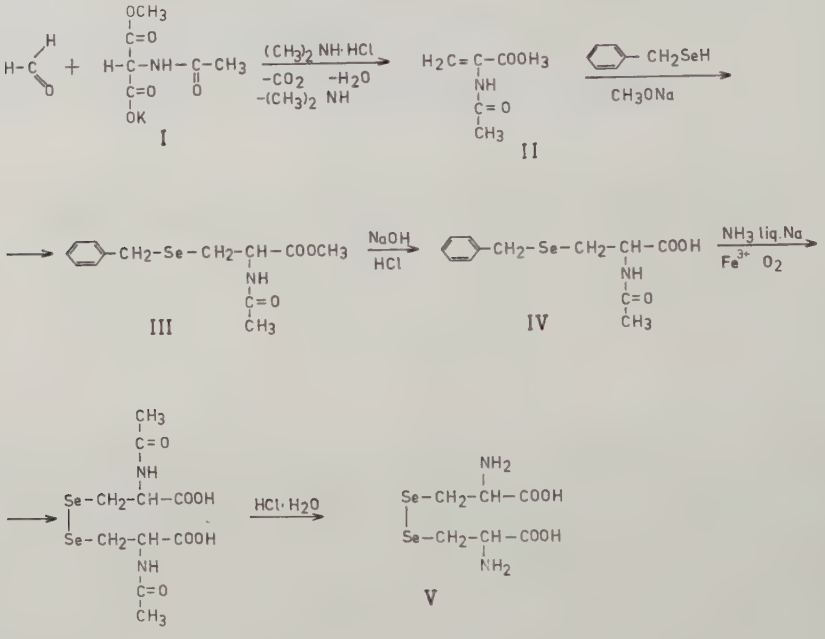
With 6 figures in the text

Ever since it became known in 1936 (Franke and Painter [1]) that selenium could enter into amino acids and proteins the selenium amino acids and their physiological effects have attracted considerable interest. Fredga [2] was the first to synthesize a selenium amino acid, *viz.* the selenium analogon to cystine.

Now that the study of the selenium amino acids has again been taken up at this institute, it has been considered desirable to find a method of synthesis that, starting from an original product cheaper than serine, would give a good yield of selenocystine. After quite a number of unsuccessful experiments with different methods of synthesis it has been decided to add benzyl selenol to an activated double bond, a method discussed by Schöberl [3] in a synoptic account of the synthesis of sulphurous amino acids for the synthesis of cystine. A similar synthesis had been carried out by Gundermann and Micheel [4] who added benzyl mercaptan to α-chloro-acrylonitrile. By autoclaving the product with liquid ammonia and subsequent hydrolysis of the nitrilo group they obtained inactive cystine after debenzylation according to Du Vigneaud. Attempts to apply this synthesis to selenocystine showed, however, that α-chloro-acrylonitrile splits off almost all selenium in free form from the benzyl selenol. Atkinson, Poppelsdorf, and Williams [5] used in 1953 diethyl α-acetamido-α-dimethylamino-methylmalonate methiodide which they made to react with sodium benzyl sulphide to give S-benzyl-DL-cysteine in 68 % yield. Later Hellmann and Folz [6] elucidated the mechanism of the reaction, and showed that α-acetyl-aminoacrylic ester is the intermediate that reacts with the benzyl mercaptan. After the publication of their elegant synthesis of α-acetyl-aminoacrylic acid esters in 1958 by Hellmann, Teichmann and Lingens [7] these esters have been used for further work.

In the course of the work in progress the sulphur analoga have been prepared in order to serve as model substances, their physical and chemical properties being in close agreement with those of the selenium compounds. The sulphur compounds are, however, in general more stable and therefore more easily handled.

In the course of these comparative preparations it has been found that benzyl selenol is added to a double bond more easily than benzyl mercaptan, if it is permissible to draw conclusions from the heat of reaction and of the duration of the first exothermal reaction. No kinetic measurements have, however, been carried out. With both benzyl mercaptan and benzyl selenol very satisfactory yields, of the same order of magnitude, have been obtained.



Experimental

The required methyl acetaminomalonate has been prepared according to Hellmann and Lingens [8], and was according to Hellmann, Teichmann and Lingens [7] transformed into *N*-acetyl-α-aminoacrylate (II). In a couple of comparative experiments II has not been isolated, but the reaction mixture utilized in its preparation has been used directly after the removal of amine and water by distillation.

S-Benzyl-N-acetylcysteine methyl ester

4.15 g of II and 3.60 g benzyl mercaptan were mixed in a 50 ml flask provided with a reflux condenser and a tube for the introduction of nitrogen. As a catalyst some drops of sodium methoxide were added. Shaking then immediately started off the reaction. The rise of temperature was moderate. The reaction mixture was heated for 10 hours in an oil bath of 100–110°. On cooling down, the entire contents of the flask crystallized. The product was dissolved in 25 ml methanol, boiled with active charcoal and filtrated. To the warm solution was added an amount of water just sufficient to initiate turbidity, whereupon the solution was allowed to cool. After some time in the refrigerator the crystals were sucked off. The compound crystallizes in thin shiny flakes. Yield 6.9 g (89 %). Melting point 84.0–84.8°. Hellmann and Folz [6] report 81°.

Analysis: Found S 11.82 %
Calculated S 11.99 %

IR see Fig. 1.

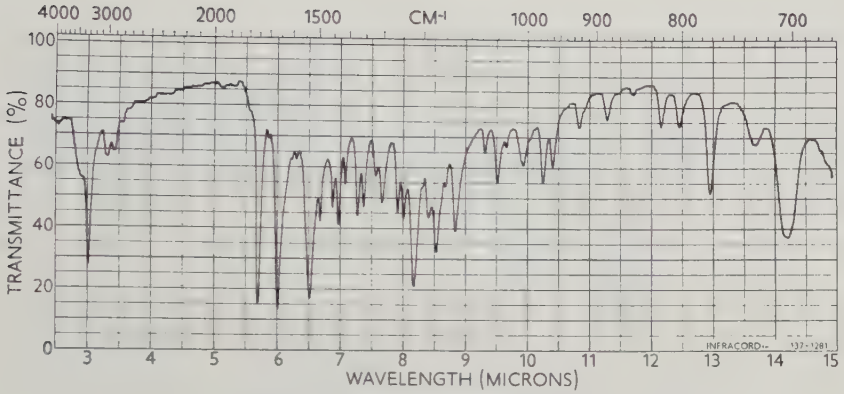


Fig. 1. IR-spectrum of S-benzyl-*N*-acetylcysteine methyl ester.

Se-Benzyl-N-acetylselenocysteine methyl ester

28.6 g of II and 34.2 freshly distilled benzyl selenol were mixed in a 100 ml two-necked flask provided with reflux condenser and a tube for the introduction of nitrogen. Some drops of sodium methoxide were added, and the mixture was shaken. After a couple of minutes the heat of reaction had raised the temperature of the mixture to about 90°. When the flask started to cool it was placed in an oil bath of 100°, where it was left for 10 hours for the completion of the reaction. After cooling the product crystallized. Recrystallization from methanol resulted in colourless plates forming starlike aggregates. Melting point 88.5–90.0°. From methanol-water the substance crystallizes in thin flakes.

Yield 59.6 g (94.7 %). IR see Fig. 2.

S-Benzyl-N-acetylcysteine

5.35 g S-Benzyl-*N*-acetylcysteine ester were heated with 20 ml 1-*N* NaOH to about 40°. Here the ester was dissolved rather quickly and the mixture was allowed

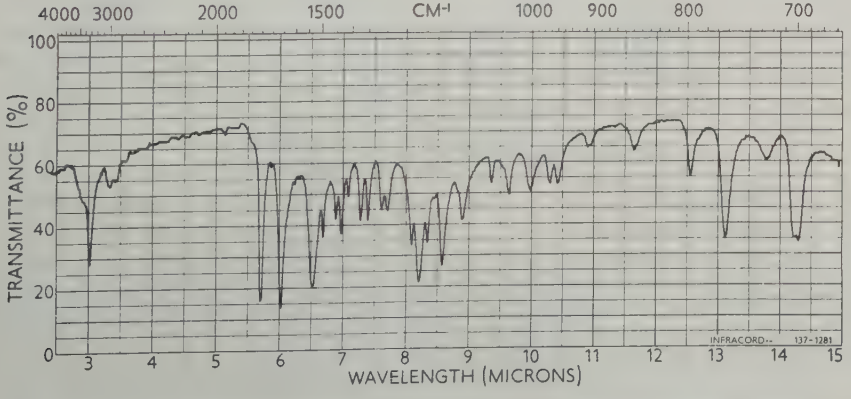


Fig. 2. IR-spectrum of Se-benzyl-*N*-acetylselenocysteine methyl ester.

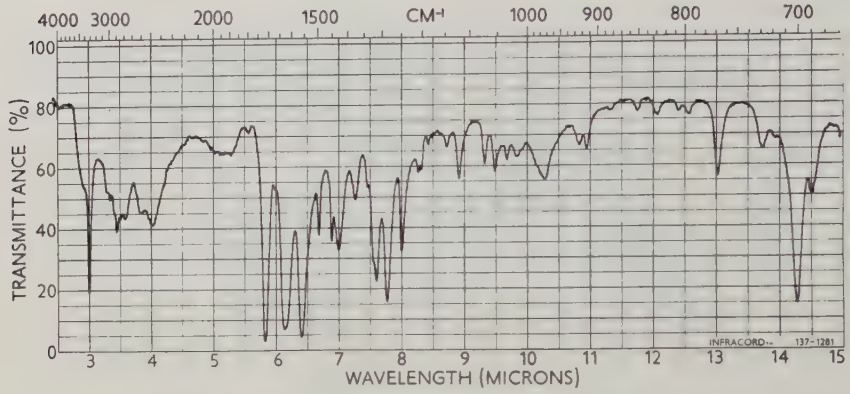


Fig. 3. IR-spectrum of S-benzyl-*N*-acetylcysteine.

to stand 6 hours in ordinary room temperature. The solution was filtered. To the filtrate were added 11 ml 2-*N* HCl, resulting in a slurry of white crystals. The mixture was put into the refrigerator over night, whereupon the crystals were sucked off. The crude product was recrystallized from 20 ml alcohol plus 30 ml water.

Yield, 4.75 g (93.7 %). Melting point, 153.5–155°. Hellmann and Folz [6] report 156–157°. IR, see Fig. 3.

0.09335 g: 7.422 ml 0.04938-*N* NaOH. Equiv. wt. 254.7. Equiv. wt. calc. 253.3.

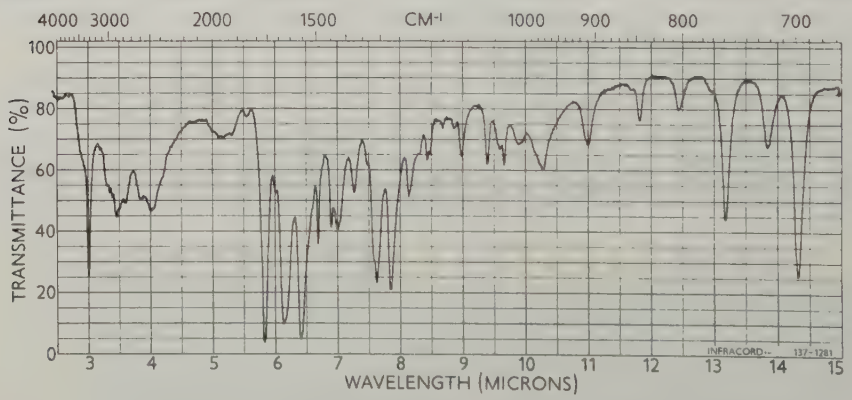


Fig. 4. IR-spectrum of Se-benzyl-*N*-acetylcysteine.

Se-Benzyl-N-acetylselenocysteine

4.29 g acylated ester were heated to 40–50° together with 16.70 ml 1-*N* NaOH, when the ester dissolved rather quickly. After addition of 5 ml water the solution was left for 12 hours in a closed vessel at ordinary room temperature. The somewhat turbid solution was extracted twice with 10 ml ether. The aqueous phase was briefly heated in order to expel dissolved ether, and its pH was brought to about 5.5 by means of dilute hydrochloric acid. This produced a copious white precipitate. After

some hours in the refrigerator the product was sucked off, washed with a small quantity of ice water, and dried in the air. 3.70 g (90.4 %) of the crude product were obtained.

The dry acid was dissolved in 20 ml of warm 95 % ethanol. Then 30 ml water were added. Recrystallisation gave 3.55 g (86.5 %) pure Se-benzyl-*N*-acetylselenocysteine in the form of colourless prisms. Melting point 152–153°. After prolonged storage, especially with exposure to light traces of red selenium render the crystals slightly pink. IR, see Fig. 4.

0.14502 g: 9.720 ml 0.04938-*N* NaOH. Equiv. wt. found 302.1 Equiv. wt. calc. 300.2.

Inactive selenocystine

1.49 g Se-Benzyl-*N*-acetylselenocysteine were suspended under stirring in about 40 ml liquid ammonia. The suspension was of yellowish white colour that became whiter, when sodium was added in small pieces. When almost all the required sodium had been added, a clear colourless solution was obtained that immediately afterwards, when the last traces of the sodium had gone into solution, became dark-blue. After the blue colour had lasted for about 10 minutes 0.4 g dry ammonium chloride were added, resulting in the immediate disappearance of the blue colour. The ammonia was allowed to evaporate through a drying tube, leaving as a remnant a honey-like mass that was dissolved in 25 ml water. pH was adjusted to 7–8 by means of hydrochloric acid, and two drops of a solution of ferrichloride were added, rendering the solution black. While the reaction mixture was stirred, air was sucked through the flask. After 40 minutes the reaction mixture suddenly became paler, and assumed a dirty, brownish yellow colour. The oxidation with air was continued for another 10 minutes. The solution was extracted with 10 ml ether. After filtration the water phase had a pure yellow colour. During the whole course of the procedure no free selenium was split off. The water phase had its pH adjusted to 5.5, and was put into the refrigerator. Since no amino acid was precipitated, 6 ml concentrated hydrochloric acid were added, and the mixture was refluxed for two hours. Then water and hydrochloric acid were removed by distillation in vacuum, the mixture being heated in a water bath. The dry remainder was dissolved in 50 ml 2-*N* hydrochloric acid that was again distilled off to dryness in vacuum. This was repeated once more. The remnant obtained in this way was dissolved in 12 ml water, and the pH adjusted against Lyphan paper to 5.5 by the addition of 2-*N* sodium hydroxide. This produced a fine-grained yellow precipitate. After 12 hours in the refrigerator the precipitate was sucked off, washed with water and alcohol, and dried in an exsiccator over silica gel. Yield, 0.72 g (87 %). IR, see Fig. 6.

The fact that the acetyl group is quantitively retained during the debenzylation gives the possibility of resolution into optical antipodes.

For comparison, a small sample of inactive cystine (IR, see Fig. 5) was prepared in the same manner as the selenocystine. This inactive cystine proved to be identical with a sample of racemized *l*-cystine.

Attempts towards the preparation of the β-benzylthio- and β-benzylseleno-α-N-acetylaminopropionic acids, respectively, from I and benzyl mercaptan, and of benzyl selenol, respectively, without isolation of the intermediate products.

13.40 g of I, 6.12 g dimethyl-ammonium chloride, and 6 ml 36 % formalin were mixed in a 100 ml three necked flask provided with stirrer, reflux condenser, and tube for the introduction of nitrogen. After 5 minutes a strong evolution of gas and a

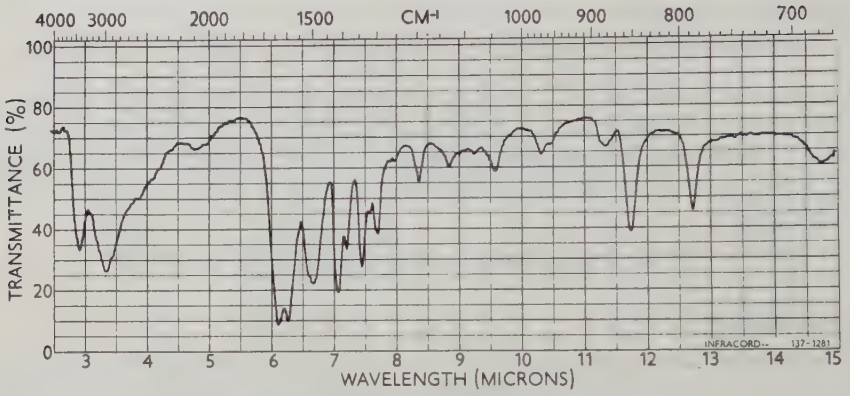


Fig. 5. IR-spectrum of inactive cystine.

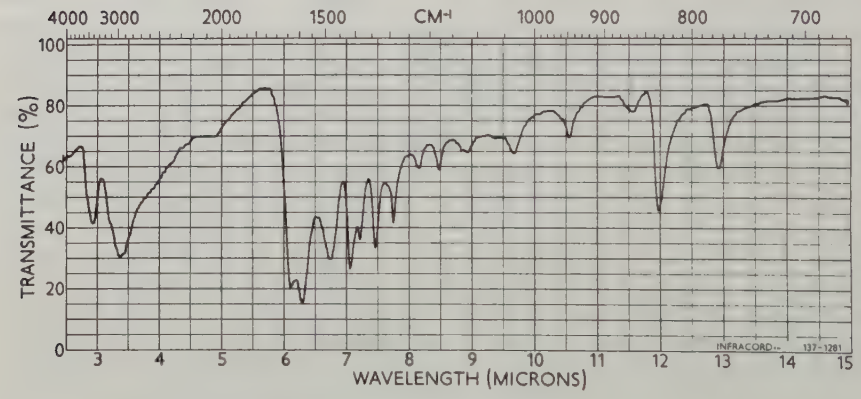


Fig. 6. IR-spectrum of inactive selenocystine.

feeble heating took place. One hour later 0.1 g hydroquinone was added, whereupon water and amine were distilled off in vacuum with simultaneous heating to 50–60° in a water bath. When all water had disappeared, the remainder became solid. In order to remove the last traces of water 30 ml benzene were added, and again distilled off. To the remainder were now added 7.85 g benzyl mercaptan, 7 ml benzene, and a couple of drops of sodium methoxide. This mixture was now heated for 5 hours in an atmosphere of nitrogen in an oil bath of 100°. The benzene was distilled off, and the remainder heated for another 2 hours. On cooling the entire product crystallized. Now, under stirring, 35 ml 2-*N* sodium hydroxide were added in which, after a while, the product dissolved. After 12 hours the alkaline solution was extracted thrice with 10 ml ether in order to remove neutral products. The aqueous phase was filtrated, and heated to 40° in order to expel the dissolved ether. Then concentrated hydrochloric acid was added so as to bring pH to 3. After 3 hours in the refrigerator the precipitate was sucked off, and recrystallized from dilute alcohol. Yield 9.92 g (62.3 %). Melting point, 153–154°.

The synthesis of the selenium analogon was carried out in the same way and with the same quantities. The benzyl selenol, 11.5 g (calc. 10.7 g), was however, not quite pure, but contained some dibenzyl diselenide. This probably accounts for the low yield. Yield, 9.50 g (51.5 %). Melting point, 152–153°. From the ether extract 4.2 g dibenzyl diselenide were isolated. Melting point, 90–91°.

ACKNOWLEDGEMENTS

Finally, I wish to express my thanks to Professor Arne Fredga, who not only directed my attention to the problems involved in organic compounds of selenium, but also always followed the progress of my work with encouraging interest. For the analyses I am indebted to Dr. Arthur Bengtsson of the Department of Analytical Chemistry, University of Uppsala.

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ARKIV FÖR KEMI Band 17 nr 25

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Induction of water-sensitivity in barley by treatment with water

By Gunnar Jansson

With 2 figures in the text

ABSTRACT

It has been established that barley can be made water-sensitive by steeping the seeds in water for 10 to 16 hours and redrying them to the original moisture content. It was found that the kind of atmosphere present during each stage had a profound influence on the extent to which water-sensitivity was formed. Thus, air (or oxygen) must be present during steeping and preferably absent during the drying procedure.

It has also been demonstrated that short periods of treatment with hot water (59–63°C) produce water-sensitivity in barley seeds. Water-sensitive barley formed in either of the two ways described here does not respond to the same degree to all chemical reagents known by earlier experiments to remove natural water-sensitivity.

Introduction

Water-sensitivity in barley seeds manifests itself by making the grains unable to germinate properly in the presence of a moderate excess of water (1). This particular form of inhibition of germination is considered to be related to seed dormancy because it disappears gradually during suitable storage of the barley (2). The natural occurrence of water-sensitivity seems to be connected to some extent with the type of weather during ripening of the grains in that it is more liable to occur in barley which ripens during wet and cold weather than in that maturing when the weather is dry and warm (3). This fact might possibly point to water-sensivity as being a natural protective measure against germination in the ear.

Methods

The germination tests were carried out according to the method described by Essery, Kirsop and Pollock (4). The amounts of water used in all experiments in the following were 4 and 8 ml unless otherwise stated. Duplicate tests were always carried out.

In the steeping experiments the barley was kept in distilled water saturated with different gases at room temperature for different times and then dried in

desiccators supplied with drying agent. When the drying was to take place in a particular kind of atmosphere, the desiccator was evacuated and the gas was let in, this procedure being repeated three times.

When the grains were treated with hot water, this was carried out in a relatively big container supplied with automatically controlled heating device. The grains were kept in muslin bags when immersed in the hot water and immediately after the end of the heating period they were cooled for about 5 minutes in ice water.

The grains which were treated with various chemicals were afterwards thoroughly washed with tap-water and adhering water was removed by drying between filter papers.

Moisture determinations were carried out by keeping the whole grains in an electric oven at 100°C until they showed constant weight.

All chemicals used were of analytically pure grade except sodium hypochlorite which was an unspecified product containing about 12 per cent active chlorine and benzoxazolone which was synthesized according to Bywater *et al.* (5).

Results

In view of the fact that water-sensitivity is more commonly found in barley seeds which have ripened under wet conditions than in seeds ripened in dry and hot weather, it was interesting to treat normal barley with water in such a way as to induce water-sensitivity in it. Barley grains were accordingly steeped in water for about 10 hours at room temperature and then dried *in vacuo* to their original moisture content as described above. It has been reported earlier (2) that water-sensitive barley recovers from dormancy after storage for some time. Barley which had recovered in this way was also treated as above in order to see whether it was more susceptible to the water treatment. Table 1 clearly shows that water-sensitivity was induced to a considerable extent in both cases and most when recovered water-sensitive barley was used.

In order to investigate the influence of different kinds of atmosphere present during steeping and drying on the induction of water-sensitivity, the seeds were steeped for 10 hours at room temperature in water which had been saturated with air or nitrogen. The grains were then dried over phosphorus pentoxide in desiccators filled with air or nitrogen at atmospheric pressure. In Table 2 the

Table 1. Effect of steeping and drying on the germination of barley seeds.

	Germination percentage after 72 hours in the presence of the following amounts of water	
	4 ml	8 ml
Normal barley, no water treatment	98	80
Normal barley, water treatment	93	26
Recovered water-sensitive barley, no water treatment	95	85
Recovered water-sensitive barley, water treatment	90	0

Table 2. Effects of different kinds of atmosphere during steeping and drying on the rate of formation of water-sensitivity.

Atmosphere during steeping	Atmosphere during drying	Germination percentage after 72 hours in the precence of the following amounts of water	
		4 ml	8 ml
Nitrogen	Nitrogen	99	63
Nitrogen	Air	100	98
Air	Nitrogen	99	33
Air	Air	97	48

Table 3. Effect of length of steep period and kind of atmosphere during steeping and drying on the rate of formation of water-sensitivity.

Atmosphere during steeping	Atmosphere during drying	Length of steeping period, hours	Germination percentage after 72 hours in the presence of 8 ml of water
Nitrogen	Nitrogen	5	86
Nitrogen	Air	5	83
Air	Nitrogen	5	85
Air	Air	5	96
Nitrogen	Nitrogen	8	80
Nitrogen	Air	8	91
Air	Nitrogen	8	79
Air	Air	8	96
Nitrogen	Nitrogen	12	82
Nitrogen	Air	12	84
Air	Nitrogen	12	5
Air	Air	12	20
Nitrogen	Nitrogen	15	88
Nitrogen	Air	15	75
Air	Nitrogen	15	22
Air	Air	15	33

results of germination tests on these seeds are tabulated and it is obvious that air (or oxygen) should be present during steeping and preferably absent during drying in order to obtain a high degree of water-sensitivity.

In a second experiment the grains were steeped for different periods of time in the presence of the same kinds of atmosphere as above, but, in order to achieve as rapid drying as possible to avoid destruction of the formed water-sensitivity, the grains were placed in drying towers and air or nitrogen was allowed to stream through the towers at a rate of 2 litres per minute. This experiment showed that a minimum time of steeping was required to obtain high water-sensitivity. The same requirements with regard to atmosphere during each step of treatment were also found as in the foregoing experiment (Table 3).

In a previous paper in this series of investigations it was reported that sodium hypochlorite solutions were effective in overcoming water-sensitivity (2). The same effect was also found in the case of water-induced water-sensitivity but consider-

Table 4. Effect of pre-treatment with sodium hypochlorite on the germination of artificial water-sensitive barley.

Concentration of sodium hypochlorite solution (active chlorine) per cent	Germination percentage after 72 hours in the presence of the following amounts of water	
	4 ml	8 ml
0.0	96	39
0.5	94	57
1.0	90	56
2.0	83	72
4.0	78	65

Table 5. Effect of pre-treatment with sodium hypochlorite on the induction of water-sensitivity by steeping.

Concentration of sodium hypochlorite solution (active chlorine) per cent	Germination percentage after 72 hours in the presence of the following amounts of water	
	4 ml	8 ml
0.0	92	30
0.5	89	8
2.0	98	74
4.0	19	0

ably higher concentrations of sodium hypochlorite had to be used and still it was not possible to achieve a complete elimination of it as appears from Table 4. The grains were steeped for 4 hours in solutions of sodium hypochlorite of various concentrations and after rinsing in tap-water the grains were germinated in the presence of 4 and 8 ml of water. In this experiment the most effective concentration of sodium hypochlorite was 2 per cent of active chlorine and in case of naturally water-sensitive barley 0.5 per cent had previously been found to be sufficient to remove the inhibition of germination (2).

In view of the above results, it appeared to be of interest to investigate whether pre-treatment with sodium hypochlorite could impede the formation of water-sensitivity by water-treatment and subsequent drying. Accordingly, barley grains were first steeped for 4 hours in sodium hypochlorite solutions of various concentrations and after rinsing they were steeped for 12 hours in aerated water at room temperature. After drying the grains were set out for germination on different amounts of water. From Table 5 it is clear that the induction of water-sensitivity was greatly reduced when the grains were treated with sodium hypochlorite at proper concentrations.

A number of sulfhydryl binding and oxidizing agents as well as some other chemical compounds (sodium nitroprusside, iodoacetic acid, N-ethylmaleimide, phenylmercuriacetate, potassium ferricyanide, hydrochloric acid) have earlier been reported to have germination-promoting effects on water-sensitive barley (2). When these compounds were applied to artificially made water-sensitive barley, no bene-

Table 6. Effect of peeling or keeping in oxygen on the germination of water-induced water-sensitive barley.

Treatment	Germination percentage after 72 hours in the presence of the following amounts of water	
	4 ml	8 ml
None	91	32
Peeling	87	85
Germination in oxygen	83	56

Table 7. Formation of water-sensitivity in barley by hot water treatment.

Pre-treatment temperature in C	Germination percentage after 72 hours of seeds (heated for various times) in the presence of 4 or 8 ml of water					
	5 min heating		10 min heating		15 min heating	
	4 ml	8 ml	4 ml	8 ml	4 ml	8 ml
51	100	99	100	100	99	99
53	100	100	99	97	98	83
55	99	99	100	98	98	75
57	100	97	99	79	95	25
59	100	88	94	23	44	0
61	100	73	68	4	7	0
63	96	32	18	0	0	0

ficial effects on the germination could be noted but the grains showed a rather great sensitivity to them when applied in concentrations in the same range or sometimes considerably below those earlier found to be sufficient and necessary to stimulate the germination of natural water-sensitive barley. Other ways of overcoming natural water-sensitivity involve germinating the grains in pure oxygen or peeling them before water is added (6, 7). When these treatments were applied to barley in which water-sensitivity had been induced, peeling was found to be the most effective treatment of the two (Table 6).

In an attempt to overcome water-sensitivity, seeds showing natural water-sensitivity were treated with hot water for short periods. It was then observed that, instead of being reduced, the water-sensitivity tended to increase considerably. Consequently, normal barley grains were investigated with respect to their response to heat treatment. The grains were immersed in hot water of varios temperatures for various times and afterwards cooled and allowed to germinate in the presence of 4 and 8 ml of water. As can be seen from Table 7 and Fig. 1, water-sensitivity appears to a considerable extent after treatment within a very narrow range of temperature.

In Fig. 2 are shown the germination figures in relation to added amounts of water in the germination test for three types of barley, namely, normal untreated (○), normal heated (◓ heated in water at 63°C for 5 minutes) and a naturally water-sensitive barley (●). The close resemblance between the germination graphs of the two last types is apparent.

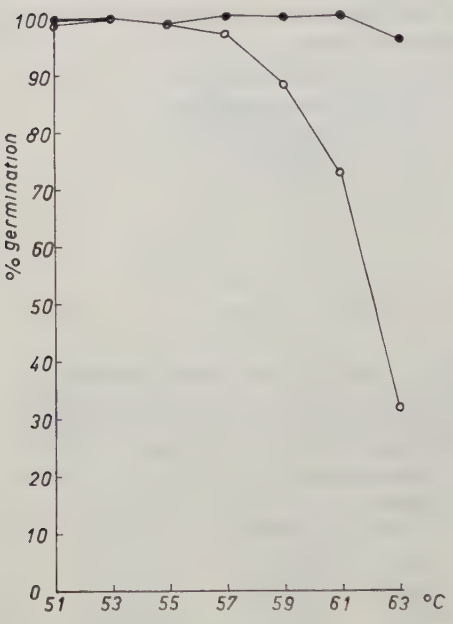

Fig. 1. Germination of barley treated with water of various temperatures (● germinated on 4 ml of water; ○ germinated on 8 ml of water).

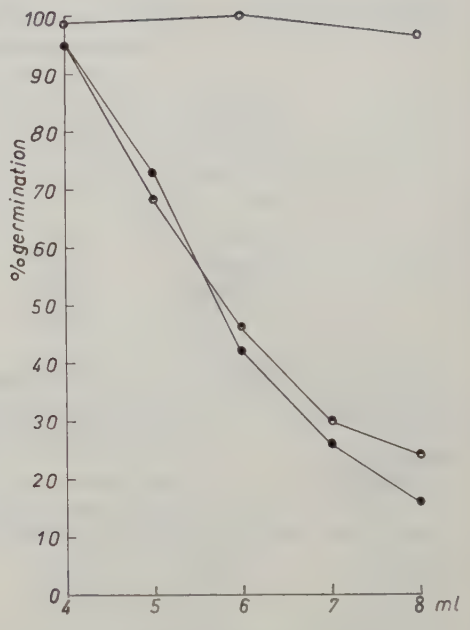

Fig. 2. Germination of barley grains in the presence of various amounts of water (○ untreated normal barley; ◓ natural water-sensitive barley; ● normal barley heated in water at 63°C for 5 minutes).

Table 8. Effect of various treatments on the germination of heat-induced water-sensitive barley.

Treatment	Germination percentage after 72 hours in the presence of the following amounts of water	
	4 ml	8 ml
None	95	18
Peeling	88	68
Steeping in 0.5 per cent NaOCl for 4 hours	91	55
Germination in oxygen	96	40

As with barley in which water-sensitivity had been formed by steeping in water, experiments were also carried out in order to try to break the heat-induced water-sensitivity with chemical agents. Normal barley grains were heated for 5 minutes in water at 63°C and afterwards the grains were steeped before germination in the same chemicals as were used earlier (2). The grains were also allowed to germinate in pure oxygen instead of air or peeled before germination. All the treated grains were set out on Petri dishes moistened with 4 or 8 ml of water. It appears from Table 8 that peeling of the grains was the most effec-

tive treatment in reducing heat-induced water-sensitivity followed by steeping in sodium hypochlorite and germination in the presence of oxygen. The other chemicals had no germination-promoting effect at all on the heat treated barley.

Discussion

In this investigation it has been established that it is possible to induce water-sensitivity in normal barley grains by treatment with water in two different ways. In one case the grains are steeped in water for a certain time and afterwards dried back to original moisture content. If such treated barley is set out for germination, a considerable inhibition of germination is observed in the presence of excessive water supply. This formation of water-sensitivity by steeping has been shown to involve three factors, namely, (*a*) presence of oxygen during steeping, (*b*) absence of oxygen during drying and (*c*) a certain length of steeping time. Water-sensitivity is induced most rapidly when oxygen is present during steeping and absent during drying. About 12 hours' steeping is needed for marked induction of water-sensitivity (Tables 2 and 3).

The other way now discovered of obtaining water-sensitivity in normal barley was to subject the grains to short treatment with hot water. The range of temperature in which water-sensitivity is formed is rather narrow (59–63°C for 5 minutes, Fig. 1) and falls within those limits between which enzymes generally are denaturated and this temperature effect might hence be due to such denaturation.

In a previous report (2) treatments have been listed which lead to overcoming of natural water-sensitivity in barley. When water-sensitive barley, formed by either of the two methods described above, was subjected to the action of the same chemical substances or treatments, it appeared that these kinds of water-sensitivity did not respond in the same manner to the treatments as did natural water-sensitivity. Peeling of the grains, germination in the presence of pure oxygen or treatment with solutions of sodium hypochlorite were found to be able to raise the germination capacity in the presence of excess of water to some extent. In the case of barley which is naturally water-sensitive peeling or germination under oxygen normally eliminate water-sensitivity while treatment with sodium hypochlorite and mercuric chloride is very effective with some samples but not with others (2, 10) (Tables 4 and 6). Other chemical substances which remove the germination inhibition of natural water-sensitive barley caused by excess of water during germination (2) do not achieve this in case of barley which is made artificially water-sensitive. Such barley rather showed an increased sensitivity against these substances with regard to their toxic properties when concentrations were used which had been applied earlier to natural water-sensitive barley. The explanation of these facts might be that the fundamental cause of water-sensitivity is differently located in naturally and artificially water-sensitive barley being situated in peripheral parts of the embryo or completely outside it in the former case, but in the latter inside the embryo.

In attempting to explain the reasons for the induction of water-sensitivity by treatment of normal barley with water in ways described in this report, it is first necessary to consider what is known of the cause of "natural" water-sensitivity. It has been suggested earlier (2, 8, 9) that the cause of water-sensitivity is the presence of an inhibitor of germination exerting its action only when the

grains are germinated in the presence of too much water. It is thought that when the grains are germinated in the presence of optimal amounts of water an oxidation reaction (probably enzymic) takes place, which reduces the amount of inhibitor to a level harmless to the grains. The explanation of the failing (or much reduced) oxidation in the grains germinated under water-sensitive conditions is considered to be a decreased oxygen supply to the enzyme system oxidizing the germination inhibitor caused by the formation of a film of water under the coats of the grains. This would obviously lead to a decrease in enzyme activity and an increase of inhibitor content consequently followed by inhibition of germination.

The experimental findings reported in this paper fit with some assumptions quite well into this hypothesis. It seems reasonable to assume that the water-sensitivity factor is present also in non-water-sensitive barley but to a smaller extent. If it now is supposed that this factor is formed during the first stage of germination (which is indicated by the experiments leading to induction of water-sensitivity by treatment with water) and that the enzymic destruction of it does not begin until a certain stage of germination has been reached, it is possible to get an explanation of the formation of water-sensitivity by steeping in water and redrying. It is conceivable that during the steeping procedure the factor is formed and accumulated to a certain level of concentration because the enzymic destruction of it has not begun at that stage of germination. If the steeping is interrupted at a convenient moment, there will be an excess of water-sensitivity factor present when the treated grains are set out for germination. This amount of the water-sensitivity factor will be additional to that formed during this second water imbibition thus reaching a level of concentration inhibitory to the grains during subsequent germination under the conditions of the water-sensitivity test but not under optimal water conditions for reasons mentioned above.

In the case of water-sensitivity induced by heat it might be supposed that the enzyme oxidizing the water-sensitivity factor will be partly inactivated and to such an extent that the oxidation will be so much impaired as to be unable to destroy the factor effectively with the oxygen supply available under the conditions of the water-sensitivity test. This will, with regard to the advanced hypothesis, lead to inhibition of germination in the presence of excess of water but not when optimal amounts of water are used.

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